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Prevalence and evolution of human parvoviruses

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ACADEMIC DISSERTATION

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No time to read, no time to sit

this is it,

another lab day to go.

Oh why, oh why, is my PCR so slow?

I've already prepared my gels, I've done quite a few

but the results under UV, I never knew.

The end of the qPCR is so near,

contamination, that's something I always fear!

The tricks of virology,

antibody assay, do I know anything about immunology?

The day of submission, sun shine,

paper accepted, time for sparkling wine.

Plasmids, hundreds of dilutions,

cloning, are there any solutions?

And what is this rapid evolution?

Writing the thesis, my eyes are almost sore

my supervisor has corrections and wants five pages more…

With the biggest thankfulness,

To all of you who made this possible

Content

Abstract

Parvoviruses are minute single-stranded DNA viruses that infect a wide range of mammalians and invertebrates. Human parvovirus B19 (B19V) was discovered in the 1970s when it caused cross-reactivity in a serological assay for hepatitis B virus. Soon B19V was found to cause several disease manifestations, including *erythema infectiosum*, arthropathy, anemias, and fetal hydrops, and in extreme cases the virus infection may lead to fetal death. The B19V titer in human blood is high during acute infection. After primary infection, B19V has been shown to persist in tissues of symptomatic and asymptomatic persons.

Prior to the commencement of this work, two new genotypes were identified for B19V that had long been considered to be very stable regarding its DNA sequence. The newly found variants are named genotype 2 and genotype 3, and the prototypic virus is genotype 1. The new genotypes were found from the blood of a child with aplastic anemia and from human skin. The sequence variation between the prototypic B19V genome and its new variants is approximately 10%. Altogether three genotypes are currently known.

The most common transmission route of B19V is the respiratory tract but transmission via plasma-derived medical products has been described. To ensure the safety of medical blood products in the European Union, new instructions were given in 2004 for B19V levels in plasma pools. These necessitate quantitative PCR (qPCR) screening for B19V DNA. Two commercially available quantitative PCR tests existed at the beginning of this thesis project and were examined here for their ability to amplify, quantify, and differentiate B19V genotypes (Study I). Both methods were optimized for the Light Cycler Instrument, and were based on fluorescence resonance energy transfer (FRET). The Light Cycler Parvovirus B19 Kit was highly sensitive for the detection of B19V genotype 1, but failed to detect B19V genotype 2 and to differentiate genotypes. The other qPCR kit proved to be a sensitive and suitable method for detection, quantification, and differentiation of all B19V genotypes, although, the sensitivity of the kit for one of the two strains of genotype 3 was low.

The prevalences of B19V genotypes were examined in a large number of blood and tissue samples (Studies I and II). B19V genotype 1 was found in 23% of maxipools of blood donor plasma (I), and in 17% of single samples of serum (II) whilst no genotype 2 or 3 DNAs were detected. Various types of tissue samples contained both B19V genotype 1 and genotype 2 DNA, while B19V genotype 3 DNA was absent from all the tissues studied (II). When grouping tissue donors according to their age, a variety in

distribution of B19V genotypes was noticed. B19V genotype 1 DNA was found in all age groups, whereas genotype 2 was confined to those subjects born before 1973. Correspondingly, sera from the past two decades contained no B19V genotype 2 DNA. The results suggest that actually the newly found B19V genotype 2 is older in occurrence than the prototypic B19V genotype 1 and it has disappeared from global circulation. Furthermore, the results indicate that persistence of B19V DNA in human tissues is lifelong and represents a source of information from our past (Study II).

The evolution rates of both persistent and acute B19V genomes were determined in collaboration with a British group at University of Edinburgh (Study III). As a sequencing target, the gene for viral capsid protein VP2, was amplified from serum samples collected from subjects with B19V acute infection. In comparison, the VP2 gene was amplified from tissues of subjects with serologically confirmed past B19V infection. Phylogenetic trees were constructed, and evolution rates were determined by linear regression and likelihood-based methods. Notably rapid sequence changes of $4x10^{-4}$ substitutions/site/year were observed in plasma-derived B19V genomes. In contrast, the evolution rate of B19Vs found in tissues was 10 times slower.

In 2005, two new human parvoviruses, human bocavirus 1 (HBoV1) and human parvovirus 4 (PARV4), were discovered by molecular screening and large-scale sequencing. HBoV1 has since been shown to cause systemic infection and respiratory illness in young children. PARV4 has mainly been restricted to those with a history of intravenous drug use with the exception of Sub-Saharan Africa. Later, in 2009 and 2010, three more human bocaviruses, probably enteric viruses, were found from feces. Whether these newly found human parvoviruses share the ability of B19V to persist in human tissues, was addressed in Study IV. Tonsillar, synovial, and dermal tissues were examined for DNAs of these new parvoviruses; neither HBoV2-4 nor PARV4 DNAs were detected. HBoV1 DNA was found in tonsillar tissue but not in synovia or skin. HBoV1 DNA prevalence was 9 % of samples collected from small children. None of the HBoV1 IgG seropositive adults harbored HBoV1 DNA. Rather than long term persistence, the results indicate a slow evanescence of HBoV1 genomes in tonsillar tissue of young children after primary exposure.

With the collaboration of a German group in Regensburg, the role of B19V DNA persistence in cardiomyopathy or myocarditis was studied (Study V). The B19V DNA prevalence was 85%. B19V genotype 1 and 2 DNAs were found in 9% and 76% of heart examined, respectively. Genotype 3 was abcent from all the tissues studied. The presence of B19V DNA in human heart biopsies demonstrated no correlation with clinical symptoms.

Quantitative PCR methods for B19V detection in plasma-derived medical products are crucial for ensuring the viral purity of the blood products. Today, quantitative B19V PCR alone, or together with antibody assays, is commonly used in diagnosis of B19V infections. Evolution studies of parvoviruses have given us a new and unexpected perspective to rates of evolution of single-stranded DNA viruses. The ability of B19V to persist lifelong in several types of human tissues is unique among parvoviruses; human bocaviruses are not suggested to occur in solid tissues for life. The detection of B19V DNA in human heart did not indicate the pathogenesis of persistent B19V, but neither answered the question about possibility of B19V reactivation. With known mechanism, and in light of full-length coding potential of the persistent viral DNA genomes, the persistence of B19V could, in future, provide the desired long-term permanence for genetherapy vectors. Furthermore, the persistence provides, at the global and epidemiological level, a database for analysis the occurrence and circulation of parvoviruses and their variants.

List of original publications

This thesis is based on the following original publications that are referred to in the text by their roman numerals. The copyright holders have given their permissions to reprint the publications.

I. Hokynar K., Norja P., Laitinen H., Palomäki P., Garbarg-Chenon A., Ranki A., Hedman K., Söderlund-Venermo M. Detection and differentiation of human parvovirus variants by commercial quantitative real-time PCR tests. J Clin Microbiol. 2004. 42, 2013-2019

II. Norja P., Hokynar K., Aaltonen L-M., Chen R., Ranki A., Partio E.K., Kiviluoto O., Davidkin I., Leivo T., Eis-Hübinger A-M., Schneider B., Fischer H.P., Tolba R., Vapalahti O., Vaheri A., Söderlund-Venermo M., Hedman K. Bioportfolio: lifelong persistence of variant and prototypic erythrovirus DNA genomes in human tissue. Proc Natl Acad Sci USA. 2006. 103, 7450-7453

III. Norja P., Eis-Hübinger A-M., Söderlund-Venermo M., Hedman K., Simmonds P. Rapid sequence change and geographical spread of human parvovirus B19: Comparison of B19 virus evolution in acute and persistent Infections. J Virol. 2008. 82, 6427–6433.

IV. Norja P., Hedman L., Kantola K., Kemppainen K., Suvilehto J., Pitkäranta A., Aaltonen L-M., Seppänen M., Hedman K., Söderlund-Venermo M. Occurrence of human bocaviruses and parvovirus 4 in human solid tissues. Submitted to J Med Virol.

V. Kuethe F., Lindner J., Matschke K., Wenzel J.J., Norja P., Ploetze K., Schaal S., Kamvissi V., Bornstein S.R., Schwanebeck U., Modrow S. Prevalence of parvovirus B19 and human bocavirus DNA in the heart of patients with no evidence of dilated cardiomyopathy or myocarditis. Clin Infect Dis. 2009. 49, 1660-1666.

Abbreviations

A6	B19V genotype 2 strain
AAV	adeno-associated virus
AMDV	aleutian mink disease virus
Anti-DIG-AP	anti-digoxigenin-alkaline phosphate
AV	adenovirus
B19V	human parvovirus B19
bp	a base pair
BKV	human polyomavirus BK
BM	Bone marrow
BPV	bovine parvovirus
BSA	bovine serum albumin
CMV	cytomegalovirus
CnMV	canine minute virus
CPV	canine parvovirus
D91.1	B19V genotype 3 strain
DIG-11-dUTP	digoxigenin-11-deoxyuridine triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EBV	Ebstein-Barr virus
EI	erythema infectiosum
EIA	enzyme immunoassay
EM	electron microscopy
ETS	epitope-type specificity
EV	enterovirus
FRET	fluorescence resonance energy transfer
FVP	feline panleukopenia virus
GE	genome equivalent
HA	hemagglutination assay
HBV	hepatitis B virus
HBoV1	human bocavirus 1
HBoV2	human bocavirus 2
HBoV3	human bocavirus 3
HBoV4	human bocavirus 4
HCV	Hepatitis C virus
HHV-6	human herpesvirus 6
HIV	human immunodeficiency virus
IC	internal control
ICTV	International Committee on Taxonomy of Viruses
IDU	intravenous drug user
IgG	immunoglobulin G
IgM	immunoglobulin M
ITR	inverted terminal repeat
JCV	human polyomavirus JC
kb	kilobase
kDa	kilodalton
LaLi	B19 genotype 2 strain
LC-PCR	Light Cycler PCR
mRNA	messenger ribonucleic acid

MCMC	Bayesian Markov chain Monte Carlo method
NP-1	nuclear phosphoprotein 1
NS1	non-structural protein
nt	nucleotide
OPD	o-phenylenediamine dihydrochloride
ORF	open reading frame
p6	B19V promoter
PARV4	human parvovirus 4
PARV5	human parvovirus 5
PCR	polymerase chain reaction
PLA_2	phospholipase 2
PPV	porcine parvovirus
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
RV	rat virus
ssDNA	single-stranded DNA
SSV	synonymous site variability
SPV	simian parvovirus
V9	B19V genotype 3 strain
VLP	virus like particle
VP1	virus protein 1
VP2	virus protein 2

Review of literature

Preface

Viruses are found wherever there is life and have probably existed since living cells first evolved. The word virus comes from the Latin word *virus,* referring to poison. A virus is a minute biological agent that consists of a genome and a protective protein shell. Some viruses also contain an envelope of membraneous structures, which protect the virus from the extracellular environment. These biological agents are obligatory intracellular parasites and share several properties with cellular life. Viruses use the host-cell components, machineries, and energy in their production of progeny viruses. Thus, it is evident that viruses have co-evolved closely with their host organisms. Similarly to their hosts, viruses undergo evolution, which helps them to adapt to environmental changes. Consequently, *Archaea*, *Prokarya,* and *Eukarya*, the domains of life, each have their own viruses.

Basic features and taxonomy of parvoviruses

Parvoviruses have non-enveloped, icosahedral capsid and linear single-stranded-DNA genome of approximately 5-6 kb. The first parvovirus was found in 1959 in rats and was named “rat virus” (RT) (Kilham and Olivier 1959). Name parvovirus was introduced in the 1960s. The name originates from the Latin word *parvum,* which means small. Parvoviruses are among the smallest viruses with a virion diameter of 18-26 nm. The structure of parvoviruses is simple; the virion consists of only proteins and genomic DNA (Tattersall 2006). Parvoviruses infect vertebrates and insects to cause systemic infections. The family *Parvoviridae* is divided into two sub-families: *Parvovirinae* and *Densovirinae (*ICTV 2009, Tattersall 2005*)*. Viruses from the sub-family *Parvovirinae* infect vertebrate cells and are divided further into five genera: *Parvovirus, Dependovirus, Erythrovirus, Bocavirus,* and *Amdovirus* (Table 1) (ICTV 2009). In addition, a sixth genus, *Partetravirus,* has been proposed. The sub-family of *Densovirinae* contains viruses of invertebrates.

The parvoviruses of *Parvovirinae* appear similar in the electron microscope (EM) and are all similar in size. The members of the different genera have differences in their hairpin structures, cellular tropism, as well as in replication and transcription strategies. The genus *Parvovirus* comprises autonomously replicating parvoviruses, which encapsidate mainly negative-strand DNA genome (Tattersall 2006). The hairpin

structures at the ends of the genome are different from each other. Dependovirus replication is dependent on co-infection; usually by adenovirus or herpes virus. Without a helper virus, the dependovirus infection remains latent. During latency, adeno-associated virus 2 (AAV2), a member of the genus *Dependovirus*, has been shown to integrate the human chromosomal DNA (Kotin et al. 1990). Dependoviruses have identical inverted terminal repeats at the ends of their genomes and encapsidate either negative or positive DNA strands in equal amounts. The viruses in the genus *Erythrovirus* infect specifically erythroid cells, from which the genus name originates. Erythroviruses also have identical hairpin structures (Deiss et al. 1990) and they encapsidate genomes with positive and negative polarities to separate virions. The *Amdovirus* genus possessed only one member for some time, the Aleutian mink disease virus, with an unusual N-terminus for viral protein 1 (VP1) (Tattersall 2006). However, in 2011 a new member of the *Amdovirus* genus was identified from gray fox (Li et al. 2011). The *Bocavirus* genus comprises of several pathogenic animal and human viruses, whose genomes encode a nuclear phosphoprotein that is unknown among other parvoviruses (Qiu et al. 2007). The terminal sequences of bocavirus genomes are different from each other. Parvoviruses infecting humans are found from the genera *Dependovirus, Erythrovirus, Bocavirus,* and the proposed *Partetravirus*.

***Table 1**. Taxonomy of parvoviruses (ICT V 2009, The Universal Virus Database).*

Subfamily	Genus	Type species
Parvovirinae	*Parvovirus*	*Minute virus of Mice, MVM* *Canine parvovirus, CPV*
	Dependovirus	*Adeno-assosiated virus AAV* *Goose parvovirus*
	Erythrovirus	*Human parvovirus, B19* *Simian parvovirus, SPV*
	Bocavirus	*Bovine parvovirus, BPV* *Human bocaviruses 1-4, HBoV1-4*
	Amdovirus	*Aleutian mink disease virus, AMVD*
	Partetravirus (proposed)	*Human partetravirus, PARV4*
Densovirinae	*Densovirus*	*Junonia coenia densovirus*
	Brevidensovirus	*Aedes aegypti densovirus*
	Iteravirus	*Bombyx mori densovirus*
	Pefudensovirus	*Periplanta fuliginosa densovirus*

Molecular features of parvoviruses - Human parvovirus B19 as an example

Parvoviruses are among the smallest viruses known and those consist only of icosahedral protein capsid and linear single-stranded-DNA genome. Human parvovirus B19 (B19V) is the type species of the *Erythrovirus* and representative member of parvoviruses. B19V was discovered when serum sample from an asymptomatic blood donor gave a false-positive result in an immunoelectrophoresis assay for hepatitis B virus (Cossart et al. 1975). The virus was detected in panel B and was coded 19, from which its name originates (Cossart et al. 1975).

Genome structure and replication of B19V

The B19V genome is a 5596 nucleotides long single-stranded DNA molecule (Cotmore et al. 1984). Identical terminal repeat structures of 383 nucleotides are located at the ends of the genome. Of these terminal structures, 365 nucleotides are imperfect palindromes, which can fold over to form hairpin structures. Hairpin structures are rich in GC pairs and the 3' -end is used as a primer in the replication of B19V (Astell and Blundel 1989, Deiss et al. 1990, Zhi et al. 2004).

In the B19V infection cycle, the virus attaches to its receptor, globoside (P-antigen), on the surface of host cells (Brown et al. 1993) and is transported into the cell by endocytosis. The P-antigen is found on the surfaces of the erythrocyte progenitor cells, endothelial, cardial, synovial, and fetal cells (Brown et al. 1993, Cooling et al. 1995). In addition to globoside, the α5β1-integrins and KU80 autoantigen function as co-receptors for B19V (Weigel-Kelley et al. 2003, Munakata et al. 2005). Inside the host cell, the B19V virion, like those of veterinary parvoviruses (Vihinen-Ranta and Parrish 2006), is transported to the nucleus (Pillet et al. 2003) where B19V DNA replication takes place. Because B19V does not encode its own polymerase, the replication depends on the host cell polymerase. In addition, the cells have to go through S phase in order for B19V to replicate.

Because the B19V terminal repeats (ITRs) are identical, it is assumed that the replication mechanism is similar to that of the adeno-associated virus (AAV) of the genus *Dependovirus*. At the first step of replication, the 3' -ITR folds and is used as a primer for complementary strand synthesis. Elongation initiates from the folded primer and is continued until the duplex DNA-molecule, which is covalently closed in the other end, has formed. Next the single strand is cut by viral NS1-protein and the opposite

original 3’ -terminus is created. The hairpin structure is melted and the original 3’-end becomes a part of the newly synthesized strand. (Berns and Hauswirth 1984, Cottmore and Tattersal 2006).

B19V has two large open reading frames (ORF) encoding nonstructural and structural proteins similar to most parvoviruses (Ozawa et al. 1987) (Figure 1). The genome also contains the third ORF that encodes a smaller nonstructural protein of 11kDa. The ORFs of B19V are present only on the negative sense DNA strand (Cottmore et al. 1986, Ozawa et al. 1987). The expression of viral proteins is controlled by a single promoter element, p6, which is located to the far left end of the genome, upstream from NS1 (Doering et al. 1987). Several TATA-boxes are located in the middle of the genome; no other promoters have been identified (Ozawa et al. 1987, Liu et al. 1991). Multiple polyadenylation signals are located in the middle and far right side of the genome (Ozawa et al. 1987, St Amand et al. 1991, Yoto et al. 2006).

Non-structural protein: The non-structural protein (NS1) of B19V consists of 671 amino acids. NS1 has a molecular weight of 77 kDa and is encoded by the left hand side of the viral genome. NS1 is located in the nucleus of the infected cell (Ozawa and Young 1987), and has several functions: it is known to be involved in viral replication, in which it binds to the 5’ -end of the genome and works as a nickase (Ozawa et al. 1987, Ding et al. 2002). NS1 binds directly or via transcription factors to the promoter p6 and acts as a transcription activator (Doering et al. 1990, Raab et al. 2002) and has also a role in inducing apoptosis by activating caspase activity (Ozava et el. 1988, Moffat et al. 1998, Sol et al. 1999, Poole et al. 2004).

Structural proteins: The right hand side of the B19V genome encodes the two structural proteins (VP1 and VP2) of B19V. VP1 and VP2 are encoded by overlapping reading frames. VP2 (58 kDa) is the major capsid protein and mediates the binding of globoside receptor, (Brown et al. 1993). VP1 (84 kDa) is identical to VP2, with the addition of 227 amino acids at the N-terminus of VP1 (Ozawa et al. 1987). The VP1 unique region contains a conserved phospolipase A (PLA_2) motif, which has been shown to be functional in B19V and in many other parvoviruses (Zadori et al 2001, Dorsch et al. 2002). The PLA_2 is most probably required for virus escape from late endosomes during viral trafficking to the host nucleus, and is thus critical for B19V infection (Zadori et al. 2001, Filippone et al. 2008). Both VP1 and VP2 contain epitopes for neutralizing antibodies (Kajigaya et al. 1991, Sato et al. 1991).

Small proteins: In addition to NS1 and VP proteins, the B19V genome also encodes two small proteins in the centre (7.5 kDa) and at the very right end of the genome (11 kDa) (Ozawa et al. 1987). Today the exact role of the 7.5kDa protein is not known while the 11kDa protein has been shown to regulate the production rates of B19V structural proteins (Zhi et al. 2006) and to induce apoptosis during B19V infection of primary erythroid progenitor cells (Chen et al. 2010).

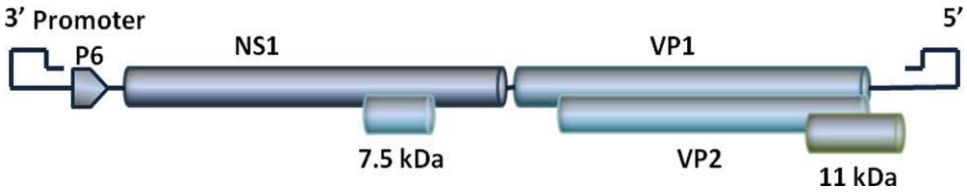

***Figure 1**. The genome structure and protein encoding reading frames of B19V.*

Genotypes of B19V

B19V strains have been divided to three divergent genotypes according to their genomic sequence (Figure 2): genotype 1 is the prototypic virus and is nowadays the most predominant circulating virus globally (Hübschen et al. 2009). The genotype 2 (LaLi- and A6-like) virus was first identified in human skin and in the serum of an Italian HIV-positive patient with chronic anemia (Hokynar et al. 2002, Nguyen et al. 2002). Genotype 2 has since been found in human solid tissues but only sporadically in blood (Nguyen et al. 2002, Schneider et al. 2004, Blumel et al. 2005, Manning et al. 2007, Kühl et al. 2008, Schenk et al. 2009, Grabarczyk et al. 2011, Koppelman et al. 2011). The genotype 3 (V9- and D91.1-like) virus was found in France in the serum and bone marrow of a child with trancient aplastic anemia (Nguyen et al. 1999, Servant et al. 2002). After its discovery, genotype 3 has been reported to be endemic in Ghana and Brazil (Candiotti et al. 2004, Sanabani et al. 2006, Freitas et al. 2008, Keller et al. 2009) and has been detected only sporadically in the Europe and the USA (Nguyen et al. 1999, Servant et al. 2002, Cohen et al. 2006, Corcioli et al. 2008, Rinckel et al. 2009).

The B19V genotypes vary by approximately 10% between each other within the genomic coding region (Hokynar et al. 2002). Within the p6 promoter, the genotypes vary more than 20% and thus p6 is the most variable area of B19V genotypes (Hokynar

et al. 2002). Despite of these differences in promoter sequences, the genotypes do not seem to have differences in their ability to infect B19V permissive cell lines (Blumel et al. 2005, Ekman et al. 2007). The transcription profiles of genotype 2 and 3 have been characterized by transfecting their respective clones to COS-7 cells (Chen et al. 2009). RNA profile of genotype 3 was identical to that of genotype 1, while the RNA profile of genotype 2 lacked one splicing acceptor. This acceptor is used to remove the first intron during transcription. Furthermore, the coding region of genotype 3 has been cloned between the ITRs of infective clone of genotype 1 and has been shown to be capable to replicate. In contrast, the coding region of genotype 2 (isolate A6) did not replicate when it was cloned between the ITRs of genotype 1 (Chen et al. 2009).

Despite differences in genomic sequences, the three B19V genotypes have similar antigenic properties. Antigenic cross-reactivity between B19V genotypes has been studied by EIAs using virus-like particles (VLPs) made of recombinant genotypes 1, 2 and 3 as antigens, and patient sera collected from individuals infected with these genotypes. All analyses showed immunological cross-reactivity between genotypes (Heegaard et al. 2002, Candotti et al. 2004, Ekman et al. 2007). In addition, the cross-neutralization experiments have shown that antibodies raised against B19V genotype 1 cross-neutralize genotype 2 in most cases (Blümel et al. 2005). No genotype-specific diseases have been described, and the genotypes 2 and 3 have been associated with clinical symptoms, such as anemia and aplastic crisis, symptoms of which are also typical to genotype 1, and therefore, are indicative of the tropism for erythroid cells (Nguyen et al. 1999, Servan et al. 2002, Sanabani et al. 2006).

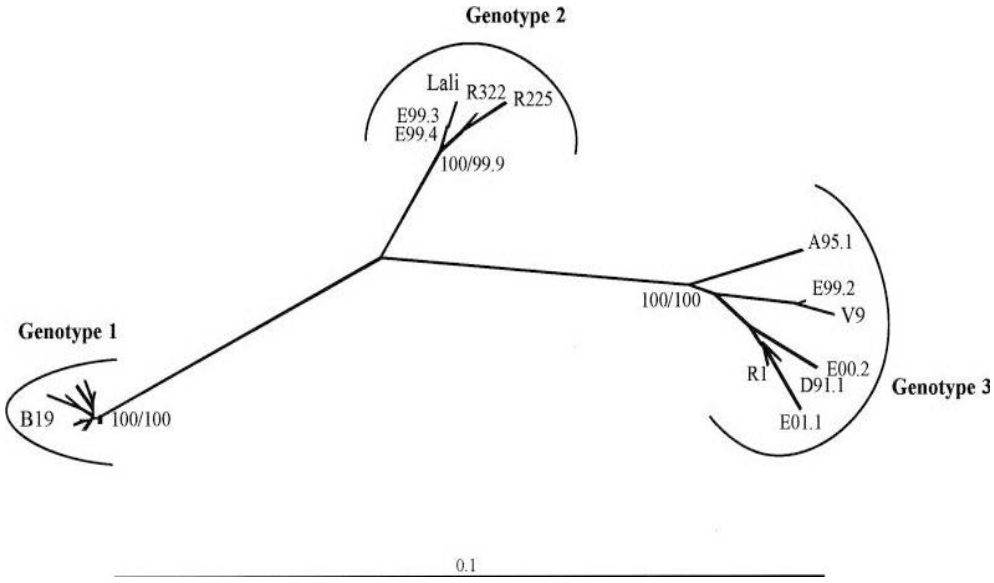

Figure 2*. The phylogenetic tree of B19V genotypes from Servant (2002), reproduced with the permission of the copyright holder.*

Transmission and epidemiology

The major route for B19V transmission occurs via respiratory droplets and contaminated surfaces, but the virus can also transmit transplacentally from mother to fetus during pregnancy (Andersson et al. 1985, Brown et al. 1984, Jordan and DeLoia 1999, Lyon et al. 1989). In addition, B19V can be transmitted via blood products (Siegel and Cassinotti 1998, Schmidt et al. 2000). The B19V titer in blood is at its highest during the first days of acute infection, when infected subjects are usually asymptomatic. This creates a risk of contaminating blood products by blood donors with asymptomatic B19V infection. Because of its small and non-enveloped structure, B19V is resistant to most viral inactivation procedures used in the manufacturing of medical blood- derived products (Willkommen et al. 1999, Koenigbauer et al. 2000, Schmidt et al. 2000). According to the European Pharmacopoeia, the blood derived transfusions manufactured after 2004 are not allowed to contain B19V DNA of more than 10^4 IU/ml. B19V DNA concentrations below this limit are not considered to lead to seroconversion (Brown et al. 2001).

B19V circulates globally and it is a significant pathogen in humans. The highest prevalence of B19V infections occurs in late winter or spring. B19V infection is most typical in children from 3 to 10 years of age (Enders et al. 2007). B19V seropositivity

rates increase gradually from 2-15% among children from 1-5 years of age to 15-60% in children and teenagers between 6 and 19 years of age and from 30-60% among adults, and it can reach as high as 80% or more among the elderly (Andersson et al. 1986, Cohen et al. 1988a, Heegaard and Brown 2002).

Disease associations

Most B19V infections are asymptomatic or mild (Harger et al. 1985, Woolf et al. 1989, Heegaard and Brown 2002). However, reticulocytopenia with a slight drop in hemoglobin concentration, occurs among subjects with symptomatic and asymptomatic B19V infection (Andersson et al. 1985). In some cases the symptoms are nonspecific and indistinguishable from those of common cold. B19V is associated with a large spectrum of diseases and most common manifestations are described below.

Erythema infectiosum

Erythema infectiosum (EI), fifth disease or "slapped cheek", is a rash disease and the most common B19V manifestation (Andersson et al. 1984). The illness is typical among B19V-infected children but adults may also be affected by it (Reid et al. 1985, Woolf et al. 1989, Harger et al. 1998). Typically, rash appears first on the cheeks, spreading then to neck, trunk, and limbs. In addition to rash, the EI patient may have headache, fever, nausea, and diarrhea. The intensity of the rash may vary and be more intense after exercise or exposure to sunlight (Naides et al. 1993). EI usually appears approximately two weeks after B19V infection, simultaneously with the appearance of B19V-specific IgM and IgG, and lasts from 1 to 3 weeks. The illness is transient and usually requires no treatment.

Arthritis

Among adults, arthritis can be the only manifestation of B19V infection affecting 45-80 % of infected subjects (Andersson et al. 1984, Reid et al. 1985, White et al. 1985, Woolf et al. 1989, Kerr et al. 1995). Arthritis is more common among women than men (Reid et al. 1985, White et al. 1985). The onset of arthritis usually appears with B19V specific antibodies and is presumably immunologically mediated. Joint symptoms are symmetrical and affect fingers, wrists, ankles, and knees (Reid et al. 1985, White et al. 1985). In some cases, arthritis may be prolonged and fulfill the criteria of rheumatoid arthritis (Naides et al. 1990, White et al. 1985). It is unusual however, that B19V arthritis causes permanent damage to joints or bones (Jawad et al. 1993).

Anemia and other hematological manifestations

Among immunosuppressed subjects with decreased ability to produce antibodies, the B19V infection may become persistent and cause chronic anemia (Kurtzman et al. 1988, Kutzman et al. 1989). Persistent B19V infections are reported among persons with leukemia and those undergoing chemotheraphy (Kurtzman et al. 1988). Transient aplastic crisis in patients with underlying hematological diseases have been described during B19V infection (Andersson et al. 1985, Heegaard and Brown 2002).

B19V during pregnancy

More than 50% of pregnant women are seropositive and thus immune to B19V infection (Harger et al. 1989, Jensen et al. 2000, Alanen et al. 2005). Women without B19V-specific antibodies are at risk of primary B19V infection and transplacental transmission. During maternal infection, the risk of vertical transmission is approximately 30 % (Brown et al. 2010). Intrauterine B19V infection has been associated with fetal anemia, hydrops, miscarriage, and fetal death (Brown et al. 1984, Enders et al. 2006). The risk of hydrops and fetal loss is predominant when infection occurs in early gestation (Miller et al. 1998, Enders et al. 2004, Riipinen et al. 2008) but has also been reported later in pregnancy (Norbeck et al. 2002).

Persistence of B19V in body parts

After primary infection, genomic DNA of B19V remains detectable in human tissues. Because one of the clinical manifestations of B19V infection is arthritis, and because symptoms in some cases fulfill the criteria of rheumatoid arthritis, synovial fluids and synovial membranes have been under wide interest for B19V investigation. Several other tissue types have also been studied for B19V DNA, and disease associations of B19V have been searched by detection of viral DNA in corresponding tissues. However, large proportion of healthy individuals also harbord B19V DNA in their body parts and thereby the mere presence of B19V DNA in tissues of a symptomatic patient does not prove that the present disease is caused by B19V. This chapter describes B19V DNA prevalences in different human body parts.

Synovia

B19V DNA has been detected, with prevalence rates varying from 5% to 75%, in synovial membranes or synovial fluids of patients with arthritis (Saal et al. 1992, Cassinotti et al. 1995, Kerr et al. 1995, Nikkari et al. 1995, Zakrzewska et al. 2001,

Schmid et al. 2007, Aslan et al. 2008). Our group collected synovial membranes from children with unexplained arthritis and from healthy young adults with joint trauma (Söderlund et al. 1997). B19V DNA was detected in 28% (8/29) of synovial membranes of children with arthritis and in 48% (13/27) of synovial membranes of healthy controls. All of the subjects studied for B19V DNA, carried B19V IgG antibodies, but no IgM or B19V DNA in serum. This study demonstrated the persistence of B19V DNA in healthy human tissue and promoted a reevaluation of the diagnostic criteria of B19V arthropathy (Söderlund et al. 1997). Later, the full coding regions of the B19V genomes were amplified in synovia (Hokynar et al. 2000). The persisting B19V sequences were compared to sequences isolated from acute-infection sera and the comparison showed no significant nucleotide changes. Thus, the B19V DNA persistence in synovia was not due to particular mutations in the B19V genome (Hokynar et al. 2000, Söderlund-Venermo et al. 2002).

Skin

B19V DNA together with capsid proteins were first detected in the skin of a patient with EI (Schwarz et al. 1994, Söderlund-Venermo et al. 2002). Later Vuorinen et al. (2002) studied the role of B19V in chronic urticaria. Samples from 36 adult patients, and 22 healthy controls were examined for B19V DNA by PCR and by serology. B19V DNA was detected in 50% of patients and in 64% of controls. Serum samples from all B19V DNA positive tissue donors were positive for B19V IgG antibodies, and no B19V IgM antibodies were detected.

In 2002, Hokynar et al. (2002) found a new B19V strain, genotype 2, in biopsies of skin obtained from constitutionally healthy adults. In skin biopsies from healthy hospital staff, or subjects with B19V-unrelated dermatological disorders, B19V DNA was detected in 41%. The variant DNA was present in the samples of skin but not in the synovia. In addition, in skin, the genotype 2 was more prevalent (47%) than genotype 1 (26%). Table 2 summarizes the results of seven studies of B19V DNA in human skin.

Table 2. *B19V DNA prevalences in skin.*

Study	Symptomatic subjects						Control subjects					
	N =	age range (mean)	B19V DNA +	gt 1 DNA +	gt 2 DNA +	gt 3 DNA +	N =	age range (mean)	B19V DNA +	gt 1 DNA +	gt 2 DNA +	gt 3 DNA +
Vuorinen et al. 2002	36		18 (50%)	nt*			22		14 (64%)	nt		
Tomasini et al. 2004	30	11-65 (32)	9 (30%)	nt			0					
Baskan et al. 2006	40	19-61 (30.5)	24 (60%)	nt			20	17-50 (36.3)	11 (55%)	nt		
Corcioli et al. 2008							38	33-93 (70)	29 (76%)	11	18	0
Sidoti et al. 2009	156	23-95 (-)	85 (54%)	29	55	6	42		17 (40%)	11	6	0
Zakrewska et al. 2009	49	20-70 (52)	29 (59%)	28	21	0	28	30-70 (55)	20 (71%)	9	11	0
Bonvicini et al. 2010	121	2-92 (-)	24 (20%)	24	0	0	24	25-80 (50.2)	6 (25%)	1	0	0

Of note: multiple genotypes were detected in the studies of Sidoti et al. and Zakrewska et al.

* nt= not typed

Bone marrow

In immunocompetent persons, B19V is usually cleared from the circulation rapidly after viremic phase. In immunosuppressed individuals, who are unable to produce neutralizing antibodies against B19V, the virus can persist in blood and bone marrow (BM) (Kurtzmann et al. 1987, Kurtzmann et al. 1988, La Monte et al. 2004). However, B19V DNA has also been detected in BM samples of some immunocompetent individuals with or without any symptoms (Cassinotti et al. 1998, Lundqvist et al. 2005). Lundqvist et al. (2005) detected B19V DNA in 26% (13/50) of B19V IgG positive and IgM negative rheumatic patients.

In two other studies, B19V DNA was detected in 8% (4/45) (Cassinotti et al. 1997) and in 2.1% (4/153) (Heegaard et al. 2002) of BMs collected from healthy bone donors or bone-marrow donors. In the first study, 3 out of 4 B19V DNA-positive subjects carried B19V IgG, and in the latter study all the B19V DNA-positive BM donors were B19V IgG positive. Later Manning et al. (2007) reported B19V genotype 1 DNA in 52% (12/23) and genotype 2 DNA in 75% (6/8) of BM samples of HIV-infected and uninfected subjects, respectively.

Garcia et al. (2011) reported B19V DNA in BM samples collected from subjects with cytopenia. For controls they also collected BM and blood samples from healthy BM donors and from oncohematological patients. The prevalences were similar; 15% (18/120) and 12.7% (21/165) among case and control groups, respectively. In the two reports, B19V genotype 2 was more prevalent in BM among older subjects than genotype 1 (Manning et al. 2007, Garcia et al. 2011). Genotype 3 was also detected in BM in the recent Brazilian study (Garcia et al. 2011).

Liver

Several studies on detection of B19V DNA in liver tissues have been published. Among the first ones, B19V DNA was detected in liver tissues of children requiring liver transplantation due to acute fulminant liver failure of unknown etiology (Langnas et al. 1995, Karetnyi et al. 1999). All of the patients carrying B19V DNA were IgG seropositive while none had IgM. B19V RNA was detected in three homogenates of 15 B19V DNA-positive livers suggesting active virus replication (Karetnyi et al. 1999). B19V DNA and RNA have also been reported by Abe et al. (2007), who suggested that B19V could be an etiological agent of fulminant hepatitis. Most of the patients in this study were liver transplant recipients with a history of blood-product infusions. Serological studies were not conducted.

In four other studies, a large number of human liver tissues were examined for B19V DNA. In the first study, B19V DNA was found in 33% (22/66) of samples from patients undergoing liver transplantation or from autopsy (Eis-Hübinger et al. 2001). In most cases the cause of transplantation was cirrhosis due to alcohol consumption or due to HCV or HBV infection. The causes of death were artherosclerosis, internal bleeding, melanoma, B-cell lymphoma, heroin intoxication, or bronchial carcinoma. All of the liver donors were B19V IgG seropositive. Later, the same group analyzed 87 transplanted liver samples for three B19V genotypes. B19V DNA was found in 59 (68%) subjects of whom 32, 27, and 4 carried genotypes 1, 2, 3 respectively. Three individual were positive for genotypes 1 and 2 (Schneider et al. 2008). Wong et al. (2003) reported B19V DNA-prevalence of 19% (7/37) in the livers of subjects with fulminant hepatitis, hepatitis-associated aplastic anemia, or nonviral hepatic disease. Significant difference in B19V DNA-prevalence between the study groups was not detected. B19V genotype 2 was detected in five and genotype 3 in two cases. Wang et al. (2009) detected B19V DNA frequently in explanted end stage liver tissues (37/50, 74%) and diagnostic liver-biopsy samples from patients with chronic liver disease (14/32, 44%). However, no association of B19V DNA and liver disease was observed in any of the four studies, but they suggested B19V persistence in human livers (Eis-Hübinger el. 2001, Wong et al. 2003, Schneider et al. 2008, Wang et al. 2009).

Heart

Cardiovascular diseases such as acute and chronic myocarditis, dilated cardiomyopathy, and coronary heart disease, are leading causes of heart failure in Western countries especially among men over 30 years of age. While it is known that human enteroviruses are among the most common causes of myocarditis (Feldman et al. 2000), parvoviruses have also been associated with cardiac diseases.

Fetal myocardial cells express P-antigen, B19V receptor, which has not been found in myocytes of older children or adults (Brown et al. 1993). Acute B19V infections are known to induce acute, in some cases fatal, myocarditis, among children and adults (Chia et al. 1996, Murry et al. 2001, Munro et al. 2003, Dina et al. 2011).

The role of B19V in heart failures has been investigated widely over the past decade, with contradictory results. In a study by Bock et al. (2010) 498 endomyocardial biopsies from adult patients with myocarditis or dilated cardiomyopathy were studied for B19V DNA. In addition, 91 heart biopsies from noninflamed hearts were analyzed as controls. B19V DNA was detected in all groups with prevalences of 64.7% in myocarditis, 35.3 % in dilated cardiomyopathy, and 7.7% among the healthy controls (Bock et al. 2010). All

of the biopsies, which contained B19V DNA, were B19V positive also in immunohistological analysis. B19V DNA quantities among the myocarditis patients were higher than among patients with dilated cardiomyopathy, or among controls. Thus the presence of B19V DNA in myocardial tissue was suggested to have a causative role in myocardial inflammation. In contrast, a study by Schenk et al. (2009) suggested no correlation between B19V DNA in heart tissues and cardiac disease. Sixty nine myocardial biopsies and blood samples were obtained during autopsy. The causes of death were craniocerebrellar injury, traumas with a blunt object, drowning, intoxication, natural death, and cardiovascular disease. No acute viral infections were suspected, and acute myocarditis was diagnosed only in one subject. In addition, the seropositivity rate for B19V IgG was 72% and for IgM 0% indicating the absence of acute infection. The serum samples from IgG-positive subjects were further analyzed by parvovirus RecomBlot-test, a commercial line-blot test, distinguishing acute and past infections; with one possible acute case. B19V DNA in the myocardial tissue was detected in 96% seropositive and 0% seronegative subjects (Schenk et al. 2009). Genotype 1 and 2 DNAs were both detected. Genotype 2 was present among subjects born between 1927 and 1957 and genotype 1 among subjects born between 1950 and 1969. There was no significant difference in prevalences of B19V DNA in myocardial tissue obtained from subjects with or without cardiac disease. The quantity of B19 DNA varied between 10^1 and 10^4 genome equivalents (GE)/µg DNA, and showed no correlation with diagnosis of acute myocarditis or cardiovascular disease (Schenk et al. 2009).

Tschöpe et al. (2005) analyzed 70 endomyocardial biopsies from persons with normal diastolic function, or with diastolic or endothelial dysfunction for B19V DNA, and found prevalence rates of 24 % and 84%, respectively. However, only 60% of B19V DNA-positive hearts were found to be inflamed, indicating B19V DNA persistence. No serology was conducted, and authors concluded an association between B19V and endothelial dysfunction.

Table 3 summarizes the B19V-DNA prevalences in 12 other studies. In several studies B19V DNA co-exists with other viruses including enterovirus (EV), adenovirus (AV), human herpesvirus 6 (HHV6), Epstein-Barr virus (EBV), or cytomegalovirus (CMV) (Lotze et al. 2004, Mantke et al. 2004, Kytö et al. 2005, Tschöpe et al. 2005, Küethe et al. 2007, Lotze et al. 2010, Stewart et al. 2010).

Table 3. *B19V DNA prevalences in heart tissue.*

Study	N =	age range (mean, years)	B19V DNA + (%)	gt 1 DNA +	gt 2 DNA+	gt 3 DNA +	B19V IgM + (%)	B19V IgG + (%)	Association to heart failure by authors
Schowengert et al. 1997	810	1 day - 21 years	9 (1%)	nt*			3		yes
Kühl et al. 2003	24	18-71 (39.5)	12 (50%)	nt				11 (46%)	yes
Klein et al. 2004	116	19-78 (52)	9 (7.7%)	nt			nd*	nd	yes
Mantke et al. 2004	110	4-76 (-)	9(8%)	nt			nd	nd	no
Lotze et al. 2004	62	23-69 (50.1)	18 (29%)	nt			nd	nd	no
Corcioli et al. 2008	19	53-84 (70)	12 (63%)	0	11	1	nd	nd	no
Kytö et al. 2005	52	7 months - 79 years	6 (11.5%)	nt			nd	nd	no
Küethe et al. 2007	216	adults	38(17.6%)	nt			nd	nd	no
Kühl et al. 2008	317	(54.3)	151 (47%)	43	108	0	nd	nd	yes, genotype 1
Stewart et al. 2010	100	adults	12 (12%)	10	2	0	0	100 (100%)	no
Lotze et al. 2010	24	21-66 (51.5)	12 (50%)	4	8	0	0%	81%	no
Ruppert et al. 2011	139	(51.9)	65 (46.8%)	38	25	1	nd	nd	yes, genotype 1

*nt indicates not typed, nd indicates not done, gt indicates genotype.

Kidney

B19V DNA has been detected by nested PCR in renal biopsies of patients with various forms of focal segmental glomerulosclerosis (85%), nephropathies (55%), as well as in normal kidney (50%) (Tanawattanacharoen et al. 2000). The prevalence of B19V DNA among healthy kidney donors has been reported in two studies by Barzon and colleagues. Among 57 donors 61% had B19V DNA prior to transplantation (Barzon et al. 2009a). After transplantation, follow-up biopsies of kidneys were obtained after 6, 12, and 24 months, and 45%, 51%, and 71% contained B19V DNA, respectively. B19V genotype 1 was the most prevalent genotype detected since only one patient demonstrated genotype 2. Blood samples from transplant recipients collected both during transplantation and follow-ups, were analyzed for B19V DNA and B19V antibodies. After transplantation, nine seronegative recipients showed persistently B19V DNA in blood (DNAemia) while five other B19V-seronegative recipients had no B19V DNAemia. All of these recipients had received a B19V DNA-positive kidney. In a second study, authors examined 75 renal transplants from 74 donors and, in addition, transplant preservation and washing solutions for viral DNA. A prevalence of 30% was detected for B19V DNA (Barzon et al. 2009b).

Brain

Animal parvoviruses have been shown to cause neuropathy when infection occurs early in neurodevelopment, which has awoken interest to examine B19V in the human brain. B19V DNA prevalence in human brain tissue has been determined in three studies. Hobbs et al. (2006) examined 104 dorsolateral prefrontal cortices, and later 105 post-mortem cerebellums, for AAV2 and B19V DNA (Hobbs et al. 2006, Grant et al. 2009). The frequency for nonpathogenic AAV2 DNA was 13% and for B19V DNA 43%, in the first study, and 26.7% for AAV2 DNA and 69.5% for B19 DNA in the second study. B19V IgG but no IgM antibodies nor B19V DNA were detected in serum of B19V DNA-positive subjects. These results ruled against acute B19V infections and suggested B19V persistence.

Manning et al. (2007) studied autopsy tissues of adults, including brain tissues of 21 HIV-infected and 8 HIV-uninfected persons, and found B19V DNA prevalence rate of 57% among HIV-infected and 100% among HIV-uninfected persons. Both genotype 1 and 2 were present in human brain tissue; genotype 2 among older tissue donors (mean birth year 1955), and genotype 1 was prevalent among younger tissue donors (mean birth year 1963-1964) (Manning et al. 2007).

Muscle

Dermatomyositis is a relatively infrequent autoimmune disease. Chevrel et al. (2000) reported B19V DNA in two muscle biopsies obtained from a patient with dermatomyositis while control biopsies from patients undergoing hip replacement contained no B19V DNA. The patient carried B19V IgG antibodies but no IgM antibodies against B19V. Later the same study group reported B19V DNA in one of eight biopsies of muscle. Most of the patients had B19V IgG antibodies (Chevrel et al. 2003).

Thyroid

Mori et al. (2007) detected B19V DNA in thyroid and peripheral mononuclear cells (PBMC) of a patient with acute B19V infection and mild hypothyroidism. The patient carried B19V DNA in thyroid for the whole four year follow up time. In study by Wang et al. (2008) B19V DNA was detected in thyroid carcinoma at a high prevalence (89.6%) and at a lower prevalence (43.8%) in controls. B19V DNA was the most prevalent among patients with papillary thyroid carcinoma (PTC) (37/38, 94.7%) and B19V capsid proteins were also detected in 63% of patients, in folliclular epithelial cells. The results pointed to a role of B19V in pathogenesis of PTC. Later Wang et al. (2010) reported B19V DNA also in 96.7% (29/32) of adults with Hashimotos's thyroiditis and in 44% (7/16) normal thyroids.

Testis

Germ cell tumors (GCT) of testis are the most common malignant tumors occurring among young men. In a study by Gray et al. (1998), forty six testis samples from patients who presented GCT of the testis, prostatic cancer with normal testis and in addition five autopsied testis samples, were examined by PCR for EBV, CMV, and B19V. Parvovirus B19V was the sole virus detected from GCT testes with a prevalence of 85% (33/39), while B19V DNA was absent from normal testes. Even if eleven out of sixteen patients with GCT testes presented B19V IgG in their sera, only one carried B19V IgM. This study initially suggested that B19V infection plays a role in testicular cancer development.

Diss et al. (1999) examined 20 malignant and 10 benign testes for B19V DNA and found virus DNA from both categories of testes (3/20 of malignant testes; 4/10 of benign testes). They concluded that B19V DNA may be present in testes, or tumor of testes, but this might not be of pathological importance. In the recent study by Ergunay et al. (2008), B19V DNA was detected in 3 out of 36 germ-cell tumors of the testis, but not in healthy testicular tissues.

Other tissues

B19 V DNA has also been detected in tonsils, adenoids, salivary gland, and spleen (Kakkola et al. 2004, Söderlund-Venermo et al. 2002, Manning et al. 2007). Kakkola et al. (2004) studied also 31 samples of bile collected during abdominal operations, but no B19V DNA was detected in this tissue type. Based on the serology, however, 45% of bile donors had previously experienced a B19V infection (Kakkola et al. 2004).

Diagnosis and treatment of B19V infections

Antibodies produced against B19V capsid proteins have proven to be a major defense against B19V infection. High titer viremia occurs during the first days of primary infection. Virus level in blood decreases when antibodies appear. IgM antibodies against VP2 or VP1/VP2 proteins are detectable from day 10 until 2 to 3 months after infection (Anderssson et al. 1986). IgG appears after the third week from infection and provides a lifetime protection against secondary infections (Brown et al. 1984).

Acute B19V infections are typically diagnosed by IgM ELISA, and PCR can also be used in the diagnosis of acute infection. Past infections are detected by IgG ELISA alone or together with PCR: the absence of B19V DNA in blood by PCR and high IgG absorbance values are marks of past B19V infection. It has been shown by chemically denatured VP protein antigens, membrane protein spotting, and conformational viral like particles that IgGs recognizing the conformational epitopes of VP1 and VP2 remain for the life, but IgGs against linear epitopes of VP2 are signs of acute infection (Söderlund et al. 1995, Kaikkonen et al. 1999, Kaikkonen et al. 2001). Especially linear heptapeptide epitope KYVTGIN have been shown to be immunodominant (Kaikkonen et al. 1999). This epitope-type specificity (ETS) can be used for timing of the infection. IgG avidity assay can also be used alone or with ETS EIA for distinguishing primary and past infections (Söderlund et al. 1995a, Söderlund et al. 1995b, Kaikkonen et al. 1999, Kaikkonen et al. 2001).

Among immunosuppressed patients, the antibody production is minimal or absent, and B19V DNA can remain detectable at low levels for months (Enders et al. 2006). Thus, ELISA or qualitative PCR alone is not a sufficient method for timing B19V infection among immunocompromised subjects. In these cases, quantitative PCR might play a role in timing the infection (Enders et al. 2006, Enders et al. 2007).

To date there are no antiviral drugs or vaccines against B19V infection. However among immunocompetent patients, treatment is not necessary and infection is self cleared. Immunodeficient patients with chronic B19V infection, and patients with transient aplastic anemia, can be treated with intravenous immunoglobulin or erythrocyte transfusions. The immunoglobulin treatment has also shown viral clearance and resolution of symptoms among patients with chronic fatigue syndrome, vasculitis, arthritis, fetal anemia, and hydrops fetalis (Rugolotto et al. 1999, Stahl et al. 2000, Enders et al. 2004, Kerr et al. 2006). However, the intravenous immunoglobulin treatment does not always lead to complete viral clearance (Liefeldt et al. 2005). Patients with arthralgia may be treated with anti-inflammatory drugs.

Newly found human parvoviruses

Modern molecular virus screening based on DNase treatment, random nucleic acid amplification, fragment cloning followed by large scale sequencing, and bioinformatic analyses, has identified many new parvoviruses from human samples (Allander et al. 2005, Kapoor et al. 2009, Arthur et al. 2009, Cheng et al. 2010, Kapoor et al. 2010a, Kapoor et al. 2011). The sample materials screened for new viruses have mostly comprised respiratory samples or feces (Allander et al. 2005, Kapoor et al. 2009, Arthur et al. 2009, Cheng et al. 2010, Kapoor et al. 2010a, Kapoor et al. 2011). The first human parvovirus identified by this approach was human bocavirus 1 (Allander et al. 2005) and later human bocaviruses 2-4, and human parvovirus 4, have been identified (Jones et al. 2005, Arthur et al. 2009, Kapoor et al. 2009, Kapoor et al. 2010a).

Human bocavirus 1

Human bocavirus 1 (HBoV1) was identified in 2005 in nasopharyngeal aspirates of children with respiratory tract infections (Allander et al. 2005). HBoV1 belongs to the genus *Bocavirus* and its closest relatives are bovine parvovirus (BPV) and minute virus of canines (MVC). With these viruses HBoV1 has approximately 40% amino acid identity within the major open reading frames.

Morphology

The structure of HBoV1 is typical for *Parvovirinae*; it is a non-enveloped, single-stranded DNA virus with icosahedral symmetry. The 3'-terminus of the genome of 5217 nucleotides encodes the nonstructural proteins NS1 (100kDa) and NS1-70 (70kDa) (Dijkman et al. 2009, Chen et al. 2010, Schildgen et al. 2012). Nuclear phosphoprotein NP1, whose function is unknown, is encoded by the middle of the genome. The 5´-terminus of the genome encodes viral capsid proteins VP1 (74kDa) and VP2 (60kDa). All of the transcripts are transcribed from a single promoter (P3) (Chen et al. 2010).

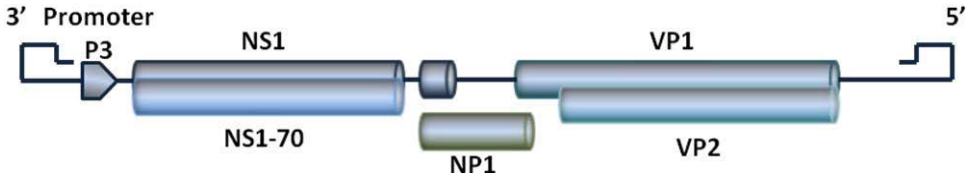

***Figure 3**. The structure of the HBoV1 genome and protein encoding reading frames*.

Diagnostics and epidemiology

The methods recommended for HBoV1 diagnosis are quantitative PCR in serum or antibody detection by IgM and IgG EIAs. Even though HBoV1-4 IgGs do crossreact, those of acute-phase, as well as HBoV1 IgM, have been shown to be HBoV1 spesific (Kantola et al. 2011). A sensitive VP2-IgG avidity assay is helpful in dating HBoV1 infections (Hedman et al. 2010, Meriluoto et al. 2012). Because of prolonged persistence of HBoV1 DNA in the airways, a qualitative PCR test in nasopharynx as such, is not sufficient for HBoV1 diagnosis.

HBoV1 infections have turned out occur early in childhood (Endo et al. 2007, Kahn et al. 2008, Lindner et al. 2008, Lin et al. 2008, Söderlund-Venermo et al. 2009, Don et al. 2009, Riipinen et al. 2010, Meriluoto et al. 2012). HBoV1 IgG seroprevalence increases until age of 6 years, when most children are seropositive (Endo et al. 2007, Don et al. 2009, Söderlund-Venermo et al. 2009, Meriluoto et al. 2012). HBoV1 IgG seroprevalences among adults have been reported to approach 100% (Endo et al. 2007, Lindner et al. 2008b, Checcini et al. 2009, Söderlund-Venermo et al. 2009).

HBoV1 DNA has been observed by PCR in respiratory secretions of young children with upper or lower respiratory tract disease, globally and throughout the year. Some studies indicate higher prevalences during winter and spring months (Allander et al. 2005, Arnold et al. 2006, Foulonge et al. 2006, Kesebir et al. 2006, Ma et al. 2006, Chung et al. 2007, Fry et al. 2007, Lau et al. 2007, Pozo et al 2007, Brieu et al. 2008, Chow et al. 2008, Cilla et al. 2008, Smuts et al. 2008). The prevalence rates of HBoV1 DNA in respiratory samples of children range from 2% to 19% (Allander et al. 2005, Foulonge et al. 2006, Bastien et al. 2007, Kesebir et al. 2006, Maggi et al. 2007, Allander et al. 2007, Fry et al. 2008, Chow et al. 2008, Tozzer et al. 2009). Among adults, HBoV1 is less prevalent and is confined to patients with immunosuppressive or cardiac disease

(Bastien et al. 2006, Manning et al. 2006, Maggi et al. 2007, Chow et al. 2008, Longtin et al. 2008, Costa et al. 2009, Ringhousen et al. 2009).

HBoV1 DNA has also been found in serum, feces, urine, saliva, and cerebrospinal fluid (Pozo et al. 2007, Chieochansin et al. 2008, Tozzer et al. 2009, Söderlund-Venermo et al. 2009, Martin et al. 2009, Kapoor et al. 2010, Wang et al. 2010, Christensen et al. 2010, Mitui et al. 2012), but rarely in other tissues (Lu et al. 2008, Clément et al. 2009, Falcone et al. 2011). HBoV1 has been absent in autopsied brain, bone marrow, and lymphoid tissues (Manning et al. 2007). The same holds true for tissues of deceased fetuses (Riipinen et al. 2010). However, adenoid and tonsillar tissues of young children showed HBoV1 DNA prevalences from 5% to 32% (Lu et al. 2008, Clément et al. 2009). HBoV1 was also reported in 17.6% of paranasal sinuses or nasal polyps, predominantly in adult patients undergoing elective surgery for chronic sinusitis (Falcone et al. 2011).

Clinical features

The clinical role of HBoV1 has been under active investigations during the past few years. Initially, HBoV1 DNA was found much more frequently in children with respiratory symptoms than in asymptomatic subjects, but later HBoV1 DNA has also been found prevalently among the latter (Kesebir et al. 2006, Allander et al. 2007, Fry et al. 2007, Maggi et al. 2007, von Linstow et al. 2008, Martin et al. 2010, Söderlund-Venermo et al. 2009). HBoV1 DNA was reported in 43% of children who had undergone tonsillectomy, indicating prolonged occurrence of HBoV1 DNA in asymptomatic children (Longtin et al. 2008). Characteristic feature of HBoV1 is that it has frequently been detected in co-infections with other respiratory viruses such as respiratory syncytial virus (RSV), rhinovirus, influenza virus, and adenovirus (Allander et al. 2005, Sloots et al. 2006, Allander et al. 2007, Fry et al. 2007, Pozo et al. 2007, Vicente et al. 2007, Brie et al. 2008, Cilla et al. 2008).

HBoV1 has been detected in 1-9% of stool samples from children with symptoms of gastroenteritis (Albuquerque et al. 2007, Lau et al. 2007, Vicente et al. 2007, Yu et al. 2008, Szomor et al. 2009, Chow et al. 2010). From 20% to more than 50% of these children were also infected with other viruses causing enteric diseases (Albuquerque et al. 2007, Lau et al. 2007, Vicente et al. 2007, Yu et al. 2008, Sozmor et al. 2009, Chow et al. 2010). In most of these studies, HBoV1 occurred in both respiratory samples and stool. The significance of HBoV1 as enteric virus is questionable since there is lack of evidence of replication in the enteric tract or firm clinical association to gastric disease.

Combined serological and PCR analysis of HBoV1 showed HBoV1 DNA in serum of 94% of wheezing children with HBoV1 serodiagnosis (Söderlund-Venermo et al. 2009). In other studies, HBoV1 serodiagnosis and HBoV1 DNA in serum correlated with respiratory symptoms including pneumonia and otitis media (Allander et al. 2007, Kantola et al. 2008, Karalar et al. 2010, Don et al. 2010, Meriluoto et al. 2012). According to the most recent longitudinal study, primary infections of HBoV1 are significantly associated with respiratory illnesses (Meriluoto et al. 2012). A correlation between high HBoV1 DNA concentration in respiratory samples and clinical symptoms has also been observed (Söderlund-Venermo et al. 2009, Christensen et al. 2010).

Human bocaviruses 2-4

In 2009, human bocavirus 2 (HBoV2) was discovered by PCR in stool samples of patients with acute flaccid paralysis in Pakistan, and at lower frequencies from Scottish adults and children with enteric disease (Kapoor et al. 2009). Soon thereafter, researchers in Australia found HBoV2, as well as a new human bocavirus, HBoV3, in children with diarrhea (Arthur et al. 2009). In 2010, Kapoor et al. were screening stool samples from several countries and found all three previously found HBoVs, and, in addition, a new HBoV species, HBoV4 (Kapoor et al. 2010).

Similarly to HBoV1, the genomes of HBoV2, 3, and 4 encode three major ORFs. The ORF at the 3' -side encodes the non-structural protein NS1, the middle ORF encodes NP1, and the 5' -side ORF encodes the overlapping capsid proteins VP1/VP2 (Kapoor et al. 2010). In genomic analysis, HBoV2 and HBoV1 show of 67% to 80% nucleotide homology (Kapoor et al. 2009). The HBoV3 genome shows close homology to HBoV1 in the nonstructural protein coding regions NS1 and NP1 (87% nucleotide similarity), but is more similar to HBoV2 in the structural protein-coding region VP1/VP2 (77% nucleotide similarity) (Arthur et al. 2009, Kapoor et al. 2010). This suggests that HBoV3 may have arisen by recombination of HBoV1 and HBoV2. HBoV4 was shown to be a recombinant of HBoV2 and HBoV3 (Kapoor et al. 2010).

HBoV2 has been found in stool and respiratory samples while HBoV3 and 4 have been detected in stool samples. HBoV2 seems to be the most prevalent of the enteric HBoVs, causing gastroenteritis and circulating globally (Arthur et al. 2009, Blinkova et al. 2009, Han et al. 2009, Kapoor et al. 2009, Shan et al. 2009, Chow et al. 2010, Kantola et al. 2010, Kapoor et al. 2010, Song et al. 2010, Wang et al. 2011). The virus-like particles of HBoV1-4 have been shown to cross react, which affects the serological assays of these viruses (Kantola et al. 2011).

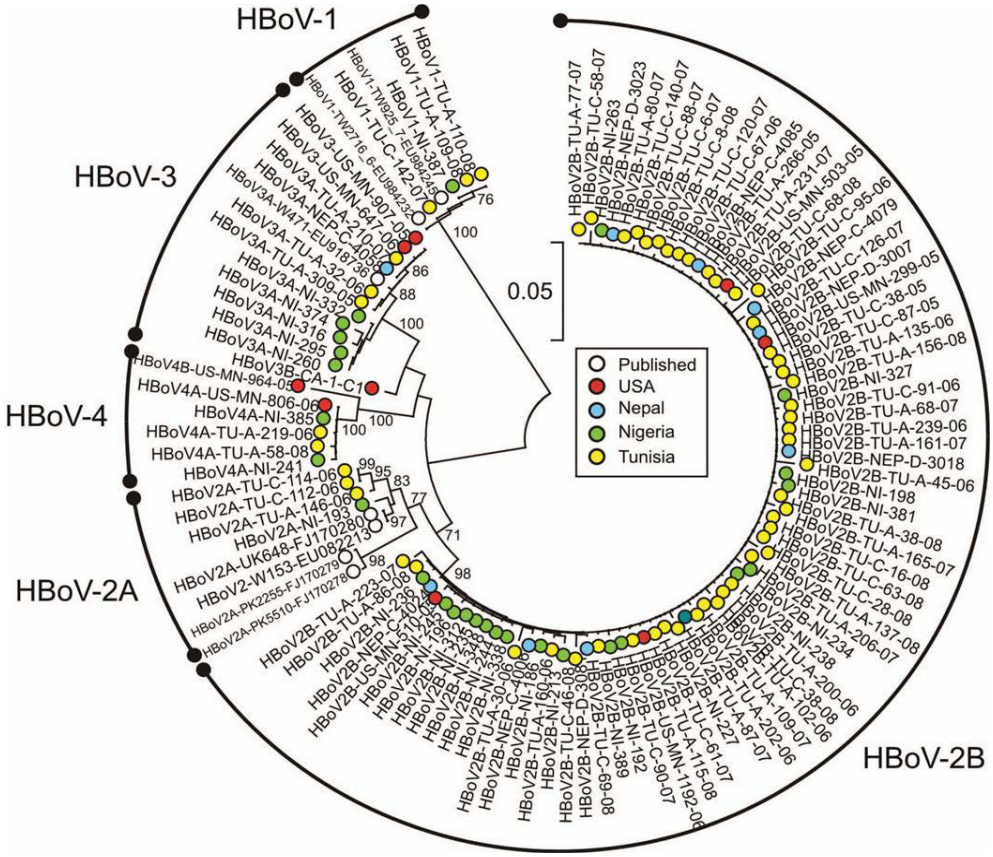

Figure 4. *Phylogenetic tree of human bocaviruses. From Kapoor (2010), reproduced by permission of copyright holder.*

Human parvovirus 4

Parvovirus 4 (PARV4) was identified first in 2005 by molecular screening and large scale sequencing approaches from hepatitis B-positive drug user (IDU) with various symptoms (fatigue, vomiting, arthralgias, neck stiffness, night sweats, and diarrhea) (Jones et al. 2005). A related virus variant (PARV5) was identified in plasma pools used in the manufacturing of plasma-derived medicinal products (Fryer et al. 2006). Later, the name PARV5 was changed to PARV4 genotype 2. In 2008, a third genotype of PARV4 was found from two AIDS patients in sub-Saharan Africa (Simmonds et al. 2008).

PARV4 is not closely related to any of the previously known parvoviruses in the genera *Parvoviridae*, but represents a lineage between avian dependoviruses and bovine parvovirus type 3 (Jones et al. 2005). PARV4-like viruses have been detected in swine and cows in Hong Kong and Great Britain (Lau et al. 2008, Szelei et al. 2010). These viruses are putatively named porcine and bovine hokoviruses (Lau et al. 2008). PARV4-like viruses are also found in plasma of Cameronian wild chimpanzees and gorillas (Sharp et al. 2010). For PARV4 and PARV4-like viruses, a genus of *Partetraviruses* has been proposed, yet not accepted.

Morphology

PARV 4 is a nonenveloped, single-stranded, small DNA virus. The PARV4 particles are 20-22 nm in diameter and morphologically typical for parvoviruses (Tuke et al. 2010). The PARV4 genome has not yet been sequenced in full length; genomes in literature lack the terminal repeats (Fryer et al. 2007a, Lou et al. 2012). However, the genome organization and the expression profile was lately disclosed as the PARV4 based expression plasmid containing known PARV4 sequence and AAV5 inverted terminal repeats (Lou et al. 2012). The viral genome contains two promoters, p6 and p38, for transcription of NS-encoding and VP-encoding mRNAs, respectively (Figure 5). The 3' -side of the genome encodes a large nonstructural protein NS1a (66kDa). Similarly to B19V NS1, the PARV4 NS1 has been shown to induce G2/M arrest during cell cycle (Lou et al. 2012). The mRNAs for NS1b, and the predicted NS2 protein, were transcribed, however, no protein products were observed for these. The 5' -side of the PARV4 genome encodes structural proteins VP1 (101kDa) and VP2 (60kDa). The VP1 of PARV4 is especially large when comparing to VP1s of other parvoviruses. The unique part of parvovirus VP1 (uVP1) has been shown to have strong PLA_2 activity, but surprisingly, PARV4 VP1u does not exhibit PLA_2 activity (Lou et al. 2012).

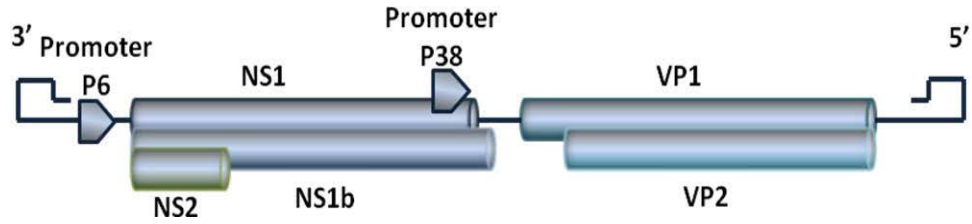

Figure 5*. The genome structure and predicted protein-encoding reading frames of PARV4*.

Diagnostics and Epidemiology

PARV4 infections can be diagnosed by IgM and IgG EIAs and PCR. Following its discovery in a person with viral acute syndrome, the DNAs for PARV4 genotype-1 and -2 have been identified in bone marrow, lymphoid tissues, and liver of subjects with a history of IDU, or HIV, or HCV infection (Manning et al. 2007, Simmonds et al. 2007, Longhi et al. 2008, Schneider et al. 2008). In several studies blood samples and coagulation factor concentrates also contained PARV4 genotype-1 and -2 DNAs (Fryer et al. 2006, Fryer et al. 2007a, Fryer et al. 2007b, Lurcharchaiwong et al. 2008, Schneider et al. 2008, Toussini et al. 2011). Two Italian studies reported PARV4 DNA in blood, skin, heart, and bone marrow of subjects without HCV, or HIV infection, or history of drug use (Botto et al. 2009, Corcioli et al. 2010). In addition, PARV4 DNA has been reported in the blood of 3 mothers having newborn with hydrops, and in the cerebrospinal fluids of two children with encephalitis (Benjamin et al. 2011, Chen et al. 2011).

For PARV4 antibodies serological assays have recently been established (Sharp et al. 2010, Lahtinen et al. 2011). A Scottish research group detected PARV4 IgG among 67% of HIV and HCV infected IDUs. Similarly, a 78% seroprevalence of PARV4 IgG was observed among IDUs, but 0% for randomly chosen Finnish medical students (Lahtinen et al. 2011). Hemophiliacs who were treated with uninactivated or virally inactivated blood products have also been shown to carry PARV4 IgG (Sharp et al. 2010, Sharp et al. 2011). The first cases and diagnostic criteria of PARV4 primary infection, and IgM-positive individuals, were recently reported among Finnish IDUs and Scottish hemophiliacs, respectively (Lahtinen et al. 2011, Sharp et al. 2011). While the latter cohort (Sharp et al. 2011) was enriched in rashes and exacerbation of hepatitis, a causal connection with PARV4 remains to be disclosed.

Evolution of parvoviruses

Genetic variation is ultimately reflected as gene mutations. Natural selection, genetic drift, gene flow, and recombination also act in evolutionary processes that shape the genetic structure of any organism. Various estimates for mutation rates of viruses have been shown (Duffy et al. 2008). These reflect the number of changes in nucleotide organization that occur during each viral replication cycle. RNA viruses are known to have high mutation rates, which are assumed to be due to error-prone RNA polymerase. Basically, a standard idea of viral evolution rates has been that most single-stranded RNA viruses mutate faster than retroviruses, which include DNA intermediates in their replication cycle, and that retroviruses mutate faster than DNA viruses (Duffy et al. 2008). The rate estimates vary between 1.5×10^{3} mutations/nucleotide/replication cycle in RNA viruses to 1.8×10^{8} for human herpes virus (large DNA virus). However, in recent studies the evolution rates of small, single-stranded DNA viruses have proven to be higher than previously believed (Shackelton et al. 2005, Shackelton et al. 2006a, Shackelton et al. 2006b, Hoeltzer et al. 2008, Streck et al. 2011).

Evolution rates of carnivore-, porcine-, and human parvovirus B19V

The best known parvovirus-related example of evolution is the transfer of a carnivore parvovirus, feline panleukopenia virus (FVP), from cats to dogs. In early 1978, a canine disease with symptoms of vomiting and diarrhea in old dogs, and myocarditis in neonatal pups, was observed globally. Small, round, non-enveloped viruses were detected in the stool of these dogs by EM, and parvoviruses were soon isolated from the tissue cultured cells. The enteric disease that the new parvovirus caused in canines was similar to that of cats caused by FPV. The new virus was shown to be antigenically related to cat parvovirus FPV and emerged from that or one of the closely related carnivore parvoviruses. The new parvovirus was named canine parvovirus (CPV) and later CPV-2 (Parrish 1999, Parrish and Hueffer 2005).

CPV-2 caused a severe pandemic with high mortality in dogs but was unable to infect cats or raccoon. However, a variant virus CPV-2a, emerged in 1979 and this virus was able to infect both cats and dogs. CPV-2a replaced CPV-2 worldwide within a year (Parrish et al. 1988). At the beginning of the 1980s another antigenic variant, named CPV-2b, was detected and was showed to spread until 1984, and was also able to infect

cats and dogs. The currently circulating virus carries mutations in the capsid protein VP2 residues 297 and 426 (Parrish 1999, Buonavoglia et al. 2000, Hoelzer et al. 2008).

The evolution rates of FPV and CPV-2 have been calculated in detail (Shackelton et al. 2005, Hoelzer et al. 2008). Shackelton et al. (2005) analyzed 91 VP2 sequences and 35 NS1 sequences of carnivore parvoviruses. A substitution rate of 9.4×10^{-5} substitutions/site/year was detected for FVP and higher rate of 1.7×10^{-4} substitutions/site/year for CPV-2. When studying the central branch of FPV and CPV separating clades containing carnivore parvovirus sequences from samples isolated between 1968 to 1978, the substitution rates ranged between 7.1×10^{-3} and 0.7×10^{-3} substitutions/site/year (Shackelton et al. 2005). These rates were similar to those detected for other RNA viruses. Later Hoelzer et al. (2008) explored the evolution of carnivore parvoviruses with a bigger sample set collected from 13 countries. The substitution rates observed were similar to those detected in the study by Shackelton et al. (2005). In addition, they showed that the majority of the substitutions distinguishing CPV from FPV were located in the capsid protein (VP2) gene in domains determining the tropism of the virus. The calculation of rates of nonsynonymous and synonymous sites and their ratios revealed that the CPV capsid gene has been under positive selection resulting in an elevated evolution rate (Hoelzer et al. 2008).

Increasing number of reports of reproductive failures due to porcine parvovirus (PPV) in swine herds encouraged a German group to study the evolution of porcine parvovirus, and evaluate the effect of a PPV vaccine against the currently circulating PPV strains (Streck et al. 2011). PPV isolates from Austria, Brazil, Germany, and Switzerland were sequenced and analyzed for their substitution rate. Their study indicated substitution rate of 5.39×10^{-5} substitutions/site/round for the NS1 gene and 3.02×10^{-4} substitutions/site/round for the VP1 gene. Further analysis showed that substitutions among new strains were mainly located at the capsid surface and capsid development towards more distinct from the vaccine strain was noted (Streck et al. 2011).

B19V has been generally considered to be a very stable virus. Erdman et al. (1996) compared B19V sequences collected from a single community and from various geographic regions. Minimal variation (0.4%) between sequences obtained from the single community, and sligly greater variation (<5%) between sequences obtained from distinct areas, were detected. No insertions or deletions were identified. Similar results have also been reported by others (Gallinella et al. 1995, Kerr et al. 1995, Hemauer et al. 1996)

Substitution rates for B19V were for the first time calculated in 2006 (Shackelton et al. 2006b). B19V sequences were collected from GenBank and aligned. The sampling dates for virus isolates were also obtained and phylogenetic trees for VP1 and NS1 areas constructed. The substitution rates 1.14×10^{-4} and 1.83×10^{-4} were detected for VP1 alone and for the whole coding region of the B19V genome (Shackelton et al. 2006b). The detected rates were very similar to those for the carnivore parvoviruses FVP and CPV (Shackelton et al. 2005, Hoelzer et al. 2008), thus suggesting that high mutation rates may be typical for all autonomously replicating parvoviruses.

Aims of the study

The specific aims of the study were:

i) to examine whether the newly found B19V genotypes 2 and 3 could be detected, amplified and differentiated by commercially available quantitative real-time PCR assays

ii) to determine the prevalence of the new B19V genotypes in Finnish blood samples

iii) to examine the persistence of B19V DNA in various human tissues and to determine the duration and possible predilection of the tissue type

iv) to study the evolution rate of B19V genomes amplified from blood cells during primary infections and

v) to compare the evolution rate of B19V isolated from different tissues during persistence

vi) to study the DNA prevalence of the four human bocaviruses and parvovirus 4 in human tissues

vii) to examine whether the occurrence of B19V DNA in heart tissue has a role in etiology of heart diseases

Materials and methods

Clinical material

Tonsillar tissue (II, III and IV): Altogether 443 tonsillar tissues were collected in Finland, from patients with tonsillitis, peritonsillar abscess, snoring, or tonsillar hypertrophy. The biopsies were collected in two separate sets. A set of 220 tonsillar tissues was collected 2000 to 2002 from children and adults (birth year range, 1929-2000; mean, 1979 ± 15) and examined in Studies II and IV. Another set from 223 children (birth year range, 1991-2005; mean, 1999 ± 4) was collected 2000 to 2006 and were examined in study IV. B19V genotype 1 DNA-positive tonsils (N=14) collected from adults were included in Study III.

Synovia and skin pairs (II, III and IV): Biopsies of synovium (N=86; birth year range, 1931-1992; mean, 1964 ± 15) were obtained during arthroscopy from healthy adults with joint trauma, and biopsies of skin were obtained simultaneously from the arthroscopy wound edge. All synovial and skin pairs were collected in Finland. B19V-genotype-1 DNA positive skin (N=1) was included in Study III.

Skin (I, II and IV): Biopsies of skin (N= 54; birth year range, 1913–1991; mean, 1951 ± 19) were obtained from patients with B19V-unrelated dermatological lesions or from healthy laboratory or hospital staff. All skin biopsies were collected in Finland.

Liver (II): Biopsies of liver tissue (N= 77; birth year range, 1915–1981; mean, 1948 ± 14) were collected from adults in Germany for diagnostic purposes and from transplantation of explanted livers. These tissues were examined in Study II for B19V genotypes 1 and 2 DNA in Germany.

Bone marrow and lymphoid tissue (III): Autopsy samples of bone marrow and lymphoid tissue (N=14) were collected between the years 1990 and 2007 in Edinburgh, Scotland. All tissues were from adults.

Heart tissue (V): Biopsies of left atrium were obtained from 100 adult patients who underwent open-heart surgery. The surgical procedures included valve replacement or correction and coronary artery bypass grafting alone or in combination. The biopsies were performed in Germany 2007 to 2008 and were analyzed mostly in Germany. The B19V genotyping was performed in Finland.

Serum (II, IV, V): Serum (group I) was collected from tissue donors for serological analysis (Studies II, IV, V). Study II also included 1640 (birth year range, 1907-1993; mean, 1966 ± 19) sera collected in Finland for virus diagnosis (group II). Among the 1640 sera, 1393 were collected from patients with rash, fever, or other constitutional symptoms and initially screened for rubella, measles, sindbis, or hantaviral disease. In addition, sera (N=247) were obtained from patients with serologically confirmed *erythema infectiosum*.

Plasma (I, III): Plasma samples (N=140 160 samples in 292 maxipools) obtained from Finnish blood donors were collected at the Finnish Red Cross Blood Service during the year 2002. The plasma samples were prescreened for B19V genotype 1 DNA as maxipools of 480 units / pool by the Parvovirus B19V quantification Kit by Roche (qPCR A). In addition, 11 of high-titer B19V DNA singe donation plasmas were collected 2002 to 2003 at the Finnish Red Cross Blood Service (Study I). A collection of 33 plasma samples, taken 2002 to 2007 from patients with B19V acute infection, or plasmas from archived plasma pools, obtained in 1990 or earlier, were collected from Finland, the United Kingdom, and Germany (Study III).

Plasmid clones

pB19: The plasmid clone of B19V genotype 1 covering nucleotides 180-5416 has been described in Brunstein et al. (2000) The genotype 2 genome was amplified by PCR from skin in several overlapping areas (LaLi 1-6), and cloned (Hokynar et al. 2002). Constructs containing LaLi1, LaLi2, LaLi3 and LaLi6 were used in validation of the quantitative B19V PCRs. After several restriction and ligation steps, a single clone covering nucleotides 105-5147 was constructed and used as a positive control in other B19V PCRs. A genotype 3 V9-clone, covering nucleotides 282-5314, and a genotype 3 D91.1-clone, covering nucleotides 1-5028, were kindly provided by A. Garbarg-Chenon, France. These clones were used for validation of B19V quantitative PCR assays and as positive controls in the qualitative PCRs (Studies I, II, V). Nucleotide numbering was according to the GenBank sequence entry AY504945. Table 4 describes the backbones and GenBank numbers related to the plasmid constructs.

pHBoV1: A full-length clone of HBoV1 strain 2 was kindly provided by T. Allander, Sweden. The clone was used as a positive control in qualitative HBoV1 PCR. Ten-fold dilution series of the clone was used as quantification standard in quantitative HBoV1-PCR and in HBoV1-4 multiplex PCR (Study IV).

pPARV4: A full-length clone of PARV4 genotype 1 was kindly provided by E. Delwart, USA. The clone was used as a positive control in PARV4 PCR (Study IV).

In addition, the empty plasmids of pSTBlue-1, Litmus 29, and pcDNA2.1 were used as negative controls in the validation of the B19V quantitative PCR.

Table 4. *Plasmid constructs used in this thesis*.

Name used	Virus	Genotype	Nucleotides	Gen Bank accession	Plasmid backbone	Manufacturer of plasmid
pB19	B19V	1	1-5300	AY504945	Litmus 29	New England Biolabs, Bewerly,USA
pLaLi 1	B19V	2	105-1312	AY044266	Litmus 29	New England Biolabs, Bewerly, USA
pLaLi 2	B19V	2	1092-2332	AY044266	Litmus 29	New England Biolabs, Bewerly,USA
pLaLi 3	B19V	2	2058-3121	AY044266	pSTBlue-1	Novagen, Madison, USA
pLaLi 6	B19V	2	105-3121	AY044266	pSTBlue-1	Novagen, Madison, USA
pLali	B19V	2	105-5147	AY044266	pSTBlue-1	Novagen, Madison, USA
pV9	B19V	3	1-5028	AJ249437	pcDNA 2.1, modified	Invitrogen Life Technologies, Paisley, UK
pD91.1	B19V	3	1-5028	AY083234	pcDNA 3.1 HisB	Invitrogen Life Technologies, Paisley, UK
pST2	HBoV1	-	1-5299	DQ000496	pCRToPo	Invitrogen Life Technologies, Paisley, UK
pPARV4	PARV4	1	1-5268	AY622943	pGEM-T Easy	Promega, Madison, USA

DNA extraction

DNA from tissues collected before 2006, and from sera and plasma, was extracted by proteinase K digestion (for 30 min at +37°C for serum and plasma samples or overnight at +55°C for tissue samples) followed by phenol-chloroform extraction and ethanol precipitation. DNA from tissues collected after 2006 was extracted by the QiAamp DNA Mini Kit (Qiagen, Germany) according manufacturer's instructions for viral DNA.

DNA amplification

Due to the high sensitivity of PCR, very strict precautions were taken to avoid contaminations in all PCRs. The PCR reactions, template pipetting, DNA amplification, and agarose gel electrophoresis were performed in separate rooms. Aerosol resistant filter tips and disposable racks were used. Plasmid dilutions of appropriate viruses for each PCR and water control were used as positive and negative controls, respectively.

Quantitative B19V-PCRs (I, II):

Two commercial qPCR assays: qPCR A, Parvovirus B19 Quantification Kit, (Roche Diagnostics GmbH, Mannheim, Germany) and qPCR B, Real ArtTM parvo B19 LC PCR (Artus GmbH, Hamburg, Germany) were compared for their ability to detect and differentiate B19V genotypes (Study I). Both assays were Light Cycler-based, contained B19-specific primers and two fluorophore-labeled hybridization probes. The assays operate according to principles of fluorescence resonance energy transfer (FRET). In both qPCRs, the known concentrations of standards allowed the quantification of target DNA. Internal controls (IC) amplified by the same primers as for target amplicon, but hybridized with probes carrying different fluorophores, were included in both assays to monitor the DNA extraction and PCR process. The data were analyzed with Light Cycler Software version 3.5 (Roche Diagnostics) and by using the second derivative maximum method together with proportional base-line adjustment. In each run the WHO international standard for B19V was used as a positive control and water as negative control.

The ability of both commercial qPCRs to detect and quantify B19V genotypes was examined with serial dilutions of B19V plasmid constructs. First the quantity of the plasmid containing B19V genotype 1 was measured by qPCR A, which had previously been validated for B19V genotype 1 DNA (Hokynar et al. unpublished data). Then the DNA concentrations of each plasmid were measured spectrophotometrically,

converted to plasmid copy numbers/µl and equilibrated to contain 10^9copies/µl. To confirm the plasmid concentrations, serial dilutions of plasmids were blotted onto nylon membrane and hybridized with a digoxigenin-labeled probe against the plasmid backbone. The plasmids were then used for evaluation of the two qPCR methods.

After B19V amplification, a melting curve analysis was done to analyze the ability of the qPCRs to differentiate the B19V genotypes. The program for the melting curve analysis was: Segment 1: 95°C, hold 15s, slope 20°C/s, acquisition mode none; Segment 2: 40°C, hold time 15s, slope 20°C/s, acquisition mode none; Segment 3: 80°C, hold time 0s, slope 0.1°C/s, acquisition mode continuous. Melting points were determined with serial dilutions of B19V genotype plasmids. Each genotype alone and the mixtures of the genotypes were used for melting curve analysis.

The Real ArtTM Parvo B19 assay was used for genotyping the B19V DNA present in tissues studied in Study II.

Qualitative B19V-PCRs:

I, II, V: Biopsies of skin, synovia, tonsils and heart (I, II, V), and sera (II) were screened for B19V DNA by nested and non-nested VP1-PCRs detecting all B19V genotypes, and B19V DNA-positive samples were further analyzed for genotype 2 DNA or for genotype 2 and 3 DNAs by K71-PCR described in Hokynar et al. (2002), and by Gt3-PCR, respectively. The VP1-PCR and nested VP1-PCR are described by Söderlund et al. (1996). The biopsies of liver were examined by Gt1-PCR described by Eis-Hübinger et al. (1996), and by K71-PCR, in Germany (Study II). The reaction mixture of Gt3-PCR contained 10 x AmpliTaq GeneAmp PCR buffer I (Applied Biosystems, California, USA), 200µM of each dNTP, 0.5µM of primers and 1.25U of AmpliTaq Gold polymerase (Applied Biosystems) and water for a reaction volume of 25 µl. After initial denaturation for 10 min at 94°C, nested Gt3-PCR-program was 35 cycles at 95 °C for 30 s; at 65°C or 55 °C for 30s and at 72 °C for 30s, respectively.

III: DNA extracted from B19V acute-infection plasma or from archived plasma pools were prescreened for B19V DNA by PCR as described in McOmish et al. (1993). B19V DNA-positive samples were subjected for further amplification by three overlapping nested-PCRs, which cover the ORF2 gene of the B19V genome. The PCRs were performed by using GoTaq polymerase (Promega, Wisconsin, USA), in accordance to the manufacturer's instructions and by using the following conditions: 30 cycles, each at 94°C for 18 s, at 50°C for 21 s, and at 72°C for 3min and final extension at 72°C for 6 min. The ORF2 amplicons were sequenced from both directions by using the Big Dye

Termination Kit (Applied Biosystems). The products of sequencing reactions were analyzed in sequencing service of University of Edinburgh to obtain the final sequences.

Quantitative HBoV1-PCR (IV)

DNA from tonsillar tissues was purified with the QIAamp DNA Mini Kit, then 5 µl of purified DNA was used as a template in the HBoV1 qPCR. The qPCR was performed as described by Allander et al. (2005) except that the thermal cycler was Mx3005P QPCR System (Stratagene, California, USA), and the settings were 95°C for 10 min, followed by 45 cycles at 95°C for 15 s and at 60°C for 1 min. The 25 µl qPCR reaction mix contained molecular biology-grade water, TaqMan universal PCR master mix (Applied Biosystems), 300 nM of primers and 150 nM of probe. A ten-fold dilution series of HBoV1 plasmid was used as quantification standard.

HBoV1-4 multiplex PCR (IV)

The DNAs extracted from the 219 tonsillar tissues were examined by HBoV1-4 -specific PCR assays. Firstly, all DNA samples were examined in multiplex format, and then those with positive HBoV DNA results, were used in the singleplex PCRs detecting HBoV1, HBoV3, and HBoV2+4. Both single and multiplex PCRs were done in a volume of 25 µl using Stratagene Mx3005p (Stratagene). The singleplex reactions consisted of 1×TaqMan Universal Master Mix (Applied Biosystems) with AmpErase uracil-*N*-glycosylase (UNG), 0.6 µM concentrations of sense and antisense primers, 0.3 µM probe and molecular biology-grade water. The multiplex reactions were set up similarly, but with all five primers included. UNG was allowed to degrade any potential carryover PCR products for 2 min at 50°C before activation of the AmpliTaq Gold polymerase for 10 min at 95°C. The amplification consisted of 40 cycles of 15s at 95°C and 1min at 60°C. Each run included plasmid and no-template controls. To generate baseline-corrected fluorescence data, baseline fluorescence was automatically determined by the Mx4000 software version 3.01 (Stratagene) baseline algorithm. The cutoff for quantification cycle (C_q) determination was automatically calculated as 20 times the standard deviation of the fluorescence value of the baseline in cycles 5 through 9. Each fluorescent reporter signal from the FAM channel was measured against the internal reference dye (ROX) signal to normalize for non-PCR-related fluorescence fluctuations between samples.

Qualitative HBoV1-PCR:

Non-nested HBoV1-PCR was performed as described by Sloots et al. (2005). The 25 µl reaction mix contained 10 x AmpliTaq GeneAmp PCR buffer I (Applied Biosystems), 200µM of each dNTP, 0.5µM of primers HBoV 01.2 and HBoV 02.2, 1.25U of AmpliTaq Gold polymerase (Applied Biosystems, CA, USA), and molecular biology grade water. Initial denaturation was 15 min at 95 °C, followed by 45 cycles at 94°C for 20 s; at 56°C for 20s, and at 72°C for 30.

PARV4-PCR:

Nested-Parv4-PCR was performed as described by Fryer et al. (2007), except with some modifications made in the cycling program; at 94°C for 10 min; 40 cycles at 94°C for 20s, at 51°C, or at 56°C for 20s, and at 72°C for 20s; and extension at 72°C for 7 min. The 25µl reaction mix contained 10x AmpliTaq GeneAmp PCR buffer I (Applied Biosystems), 200µM of each dNTP, 0.5µM of primers HBoV 01.2 and HBoV 02.2, 1.25U of AmpliTaq Gold polymerase (Applied Biosystems), and molecular biology grade water.

The primer sequences for each PCR are described in Table 5.

***Table 5**. PCR primers used in the studies of this thesis.*

PCR	Primer sequence	Target virus / genotypes
VP1-PCR	p6: ggagaatcatttgtcggaag	B19V / 1,2 and 3
	p5: aggcttgtgtaagtcttcac	
Nested VP1-PCR	p6: ggagaatcatttgtcggaag	B19V / 1,2 and 3
	p3: cttctgcagaattaactgaagtc	
	p8: tgtgcttacctgtctggattg	
	p5: aggcttgtgtaagtcttcac	
K71-PCR	of: tttactgaagacaaatggaagt	B19V / 2
	or: cactgggacagttttggcaata	
	if: agtggatttcaatcaatataca	
	ir: tcataattttggcataataatag	B19V / 1
Gt1-PCR	p1: aatacactgtggttttatgggccg	
	p6: ccattgctggttataaccacaggt	
	p2: aatgaaaactttccatttaatgatgtag	
	p2: aatgaaaactttccatttaatgatgtag	
	p5: ctaaaatggcttttgcagcttctac	
Gt3-PCR	gt3f: acccattttctgtgttaacttgt	B19V / 3
	gt3r: gcgagcaactaagtcaaataa	
	gt3if: cagtgacaaatttgcccaggac	
	gt3ir: aaggattatctaaagaaatg	

PCR	Primer sequence	Target virus / genotypes
B19V evolution PCR 1	S2360: ctgccatgtgggagcttcuaatcc	B19V/ 1
	S2341: cagatttggtgcgrtgtagctgcc	
	A3809: gctgtacctcctgtacctaaaagc	
	A3793: taaaagctgaaargaactgtgttcc	
B19V evoution PCR 2	S2911: taggtatagccaactggctaag	B19V/ 1
	S3271: tgacccacgagcaccattattaaggt	
	A3940: tgggtcacctcctaatgtgtcagg	
	A3981: ggctgaattgcatggtcttcatgtgt	
B19V evolution PCR 3	S3616: ggcaaggtcargayactttagccc	B19V/ 1
	S3674: caatatgcttacttracagtrggag	
	A4825: tacgcatcytggctgagggcacg	
	A4841: ctggtgggcgtttagttacgcatc	
Parv4-PCR	pv4f: aagactacatacctacctgtg	PARV4 / 1 and 2
	pv4r: tgcctttcatattcagttcc	
	pv4if: gttgatggycctgtggttag	
	pv4ir: cctttcatattcagttcctgttcac	
HBoV1-PCR	HBoV01.2: tatggccaaggcaatcgtccaag	HBoV1
	HBoV02.2: gccgcgtgaacatgagaaacaga	
HBoV1 qPCR	fwd: ggaagagacactggcagacaa	HBoV1
HBoV1 qPCR	rev: gggtgttcctgatgatatgagc	
	probe: FAM-ctgcggctcctgctcctgtgat-BHQ	

PCR	Primer sequence	Target virus / genotypes
HBoV1-4 multiplex	HBoV1F: cctatataagctgctgcacttcctg	HBoV1
	HBoV1R: aagccatagtagactcaccacaag	HBoV1
	HBoV234F: gcacttccgcatytcgtcag	HBoV2, 3, 4
	HBoV3R : gtggattgaaagccataatttga	HBoV3
	HBoV24R: agcagaaaaggccatagtgca	HBoV2 and 4
	probe: FAM-ccagagatgttcactcgccg-BHQ	HBoV1, 2,3 and 4

Southern hybridization

In study I, serial dilutions of the B19V genotype-plasmids were blotted onto nylon membrane and hybridized with a digoxigenin labeled probe recognizing the plasmid backbone. Qualitative HBoV1-PCR results obtained in Study IV were confirmed by Southern hybridization with a digoxigenin-labeled probe. The HBoV1 probe was amplified from the pST2, with the primers for qualitative HBoV1-PCR in the presence of digoxigenin -11-deoxyuridine triphosphate (DIG-11-dUTP) (Roche GmbH). After agarose gel electrophoresis the HBoV1-PCR products were denatured in 0.4M NaOH, 1.5M NaCl, for 45 min and then transferred to Hybond N^+ nylon filter by capillary method over night, after which the filters were neutralized in PBS. DNA was dried and cross-linked to the filter at 80°C for 15 min.

Before hybridization, non-specific sites were blocked with a prehybridization solution for 30 min at 42°C, after which the hybridization solution was added and incubated overnight at 42°C. Thereafter the filter was washed in 2xSSC, 0.1% SDS at room temperature and then in 0.1% SSC, 0.1% SDS at 68°C. The filter was rinsed with buffer 1 (0.1M Tris-HCL, 0.15 M NaCl, pH 7.5) and incubated for 30 min in buffer 2 (1x TBS, 0.05% Tween, 10% Blocking Reagent; Sigma Aldrich, USA and 2.5g sucrose/10 ml). Anti-digoxigenin-alkaline phosphatase (anti-DIG-AP) (Roche GmbH,) was diluted 1:5000 in buffer 2 and the filter was incubated for 30 min in buffer 2 containing anti-DIG-AP, washed with buffer 1, and balanced in buffer 3 (0.1 M Tris-HCl, 0.1M NaCl, 0.05M $MgCl_2$, pH 9.5). Finally the filter was incubated in the dark for 1h in the substrates containing 0.0045% of 5-bromine-4-chlorine-3-indolylphosphate (BCIP) with 0.0035% of nitro-blue-tetrazolium-chloride (NBT) (Roche GmbH) in buffer 3.

Serological assays

B19V genotype 1, HBoV1-4 and PARV4 VP2 virus-like particles (VLPs) were produced in insect cells using baculovirus expression system and biotinylated (Ekman et al. 2007, Kantola et al. 2011). Non-biotinylated HBoV1-4 VP2 VPLs were also used in VLP-based competition assay.

IgM EIA (II, IV)**:** Sera diluted 1:200 in PBS-0.05% Tween 20 (PBST) were added in duplicate to goat anti-human IgM-coated (Cappel/ICN Biomedicals, Irvine, USA) microstrips for 1 h at RT. After washing, the biotinylated VP2 antigen (10ng of B19 VP2 / 60ng of HBoV1 VP2 / 20ng of PARV4 VP2) was added and EIAs were performed as described (Söderlund-Venermo et al. 2009, Lahtinen et al. 2011).

IgG EIA (II, IV): The biotinylated VP2 antigen (40 ng of B19 VP2 / 60 ng of HBoV1 VP2 or / 60 ng of Parv4 in 100 µl PBS containing 0.05% Tween 20) was added to streptavidin-coated micro strips (Thermo Fisher Scientific, USA) and EIAs were performed as previously described (Kaikkonen et al. 1999, Söderlund-Venermo et al. 2009, Lahtinen et al. 2011)

VLP-competition assay (IV): The assay was performed as described by Kantola et al. (2011). Briefly; for the detection of HBoV1 specific antibodies, the sera were diluted in PBS containing soluble unbiotinylated HBoV2+3 VLPs and incubated 1.5 hours at 4°C. The sera were transferred to wells containing immobilized heterologous HBoV VLPs. For detection of combined HBoV2/3 antibodies, the assay was performed in reverse.

Phylogenetic analysis and calculation of evolutionary rates

The ORF2-covering sequences in Study III were aligned by using Simmonic Sequence Editor 1.5. The sequence data were supplemented with 34 previously published B19V genotype-1 sequences (17 sequences from plasma/sera and 17 sequences from tissue), from which the sample type and collection year were available. The sequences amplified from plasma and tissues were analyzed separately. Phylogenetic trees of B19V ORF2 sequences were constructed by neighbor-joining of Jukes-Cantor (J-C) corrected pairwise distances between sequences. Regression analysis used maximum composite likelihood distances between each sequence and the earliest available dated sequence, collected in 1973. Distances were calculated using the program MEGA4 with an empirically determined gamma distribution value of 0.179 across all three codon positions.

A Markov Chain Monte Carlo (MCMC) approach was used to independently estimate the rate of sequence change of B19V using the BEAST package version 1.4. Output from BEAST was analyzed using the program TRACER (http://beast.bio.ed.ac.uk/Tracer).

Results and Discussion

Human parvovirus B19V is well known human pathogen identified in 1970s. After primary infection the viral DNA persists in human tissues. This thesis project was commenced in 2003, shortly after two B19V variants, genotypes 2 and 3, were identified. In the first study (I), the commercially available B19V PCR tests were evaluated for their ability to detect and differiate all three B19V genotypes. After the evaluation, the prevalences of circulating B19V genotypes among Finnish blood donors, and among subjects with virus infection-related symptoms, were examined by several PCR methods (I, II). In study II, the duration of tissue persistence and possible tissue type specificity of B19V genotypes, were studied by PCRs with a large number of human tissues. No tissue type specificity was detected, but different distribution of B19V genotypes was observed. B19V genotype 2 was prevalent in tissues of subjects born before 1973, while genotype 1 was detected in contemporary samples. B19V genotype 3 was absent from all the samples studied, indicating that it is not commonly circulating genotype in North Europe.

The identification of B19V genotypes 2 and 3, and their different distributions, have awaken the interest in the evolution studies of B19V. In study III the evolution rate of B19V genomes amplified from blood during primary B19V infection was calculated, and compared with the evolution rate of B19V genomes amplified during persistence. The evolution rate observed among sequences obtained during primary infection was ten times higher than evolution rate of persistence. The Study III also predicted years for the most recent common ancestor of B19V genotype 1 and it was dated in the 1950s. The results of Studies II and III supports the assumption that B19V genotype 1 is actually a new variant and the later identified B19V genotype 2 is an older variant that has disappeared from wide circulation.

Whether B19V DNA persistence in human heart tissue has correlation with clinical symptoms was examined in Study V, and no correlation was observed. After year 2005 several new human parvoviruses have been identified by molecular screening, large scale sequencing, and bioinformatics. DNA- and seroprevalences of the new human parvoviruses (HBoV1-4 and PARV4) were examined in Study IV to answer the question whether the tissue persistence is shared ability among parvoviruses. Human bocavirus 1 DNA was detected in 9% of tonsils of small children while human bocaviruses 2-4 and PARV4 were absent from all the tissue samples studied.

Evaluation of commercial quantitative PCR assays for detection of B19V genotypes (I)

B19V is transmitted mainly via respiratory route but spreading also occurs via medical plasma products, as B19V is a frequent contaminant in plasma products (Eis-Hübinger et al. 1999, Schmidt et al. 2001, Schneider et al. 2004, Geng et al. 2007). Several reports describe B19V infection among hemophiliacs receiving B19V contaminated coagulation factor concentrates (Yee et al. 1995, Yee et al. 1996, Matsui et al. 1999, Blümel et al. 2002). At the beginning of this thesis project, two new B19V genotypes, genotypes 2 and 3, had been identified. In addition, the new instructions for B19V concentration in plasma pools used for human anti-D immunoglobulin production were set by the European Union (European Pharmacopoeia 2004). Quantitative PCR method for B19V detection was, therefore, needed in the manufacture of blood-derived medical products. Study I compared the performances of commercially available qPCR assays, qPCR A and qPCR B, for the ability to detect, quantify, and differentiate B19V genotypes 1, 2 and 3.

qPCR A was highly sensitive for the detection of B19V genotype 1. The limit for accurate quantification of genotype 1 was 50 copies/reaction. The qPCR A barely recognized B19V genotype 2, however: in four parallel runs, only two, gave a positive signal, with > 5 million copies of pLaLi1/reaction. Further diluted preparations of pLaLi1 remained negative. Similar results were obtained with pLaLi 6, which contained the full NS1 region of the B19V genome. qPCR A recognized one of the two genotype 3 isolates. Isolate V9 was detected with a sensitivity of approximately 1 log lower than genotype 1. The signal for genotype 3 isolate D91.1 remained negative, even with a plasmid dilution of $1x10^9$ copies/µl. Melting curve analysis of qPCR A showed the same two peaks for all amplicons regardless of genotype (Figure 6). Thus qPCR A is not sufficient for the differentiation of the three B19V genotypes.

qPCR B, in contrast, detected and amplified genotypes 1 and 2, and isolate V9 of genotype 3, with equal sensitivity. All three were detected to copy numbers as low as 5/reaction. However, the sensitivity for the genotype-3 isolate D91.1 was approximately 3 log lower than for V9, and genotypes 1 and 2. The detection limit for D91.1 was 5000 copies/reaction. Melting curve analysis after qPCR B amplification, gave distinct peaks for each genotype (Figure 6). The mean melting temperatures for genotype 1, genotype 2, and genotype 3 were 67.57°C, 65.34°C, and 60.96°C, respectively. When the genotypes were tested in mixture of two or three genotypes per reaction, mimicking dual or triple infections, the individual melting peaks were not

always observed. A mixture of all three genotypes gave melting peaks characteristic for genotypes 1 and 3. A mixture of genotype 1 and 2 resulted in only one peak that was characteristic for genotype 1. A mixture of genotypes 2 and 3 showed two correct peaks. As demonstrated with the plasmid dilution series the melting temperatures did not shift upon variations in DNA template loads (Figure 6).

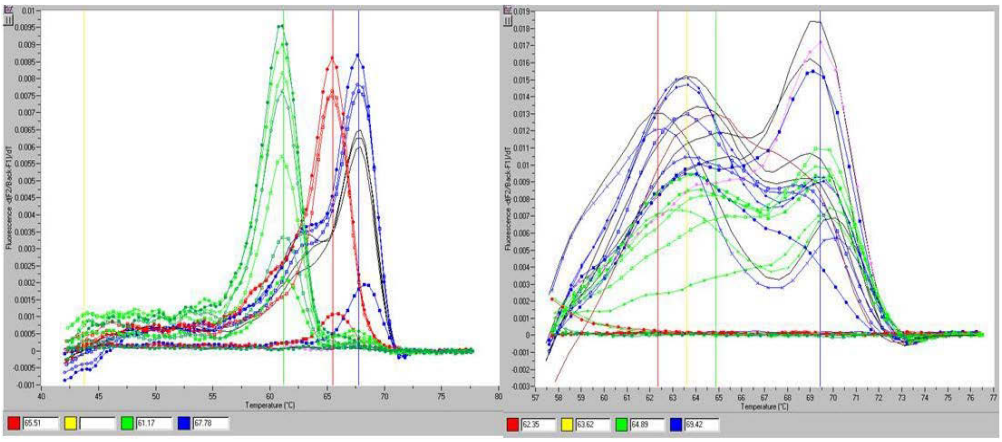

Figure 6. *The result of melting curve analysis of qPCR B (left) and qPCR A (right).*

The suitability of qPCRs A and B for the identification of the three B19V genotypes in clinical samples was examined with skin biopsies containing genotypes 1 (4 biopsies) or 2 DNA (6 biopsies), and with serum samples containing genotype 3 (2 sera). Because of the low B19V DNA level in these samples, and the presence of inhibitory compounds in the phenol-chloroform extracted DNA preparations, both undiluted and 1:10 diluted samples were tested. qPCR A detected samples containing B19V genotype 1 DNA whilst all biopsies that contained B19V genotype 2 DNA, as well as genotype 3 DNA-positive serum samples, gave negative results. The internal control (IC) gave a positive signal indicating the absence of PCR inhibition. In line with the results obtained with plasmids, the qPCR B also detected all genotypes in clinical samples. However, the serum containing genotype 3 isolate V9 remained negative, even though IC was positive. The three genotypes in clinical samples could be differentiated by melting curve analysis with qPCR B.

In summary: qPCR A was slightly more sensitive for detection of B19V genotype 1 than qPCR B. However, the ability of qPCR A to detect B19V genotypes 2 and 3 was not desirable, while qPCR B performed well. qPCR B detected all the B19V genotypes with high sensitivity, with the exception of the B19V genotype-3 isolate D19.1 that was detected with lower sensitivity. qPCR B detected all B19V genotypes also in clinical

samples and allowed the differentiation of genotypes by melting curve analysis. The performance of qPCR A with clinical samples or in melting curve analysis was different and showed no genotype 2 detection in biopsies nor the genotype differentiation capability by melting curve analysis.

Soon after Study I, the same qPCR methods were evaluated with B19V genotype-containing plasmids and compared to TaqMan- and SYBR Green 1- based in-house B19V qPCRs (Baylis et al. 2004). The results were identical to Study I. The specificity of qPCR A to B19V genotype 1 was good, but it was the only genotype to be detected. qPCR B detected and quantified all B19V genotypes, but with reduced sensitivity for isolate D91.1. Melting curve analysis was not performed in that study. Braham et al. (2004) evaluated qPCR A for B19V genotype 1 diagnostics and compared the commercial assay with an in-house B19V nested PCR and hybridization (Braham et al. 2004). Altogether 228 clinical samples were examined and qPCR A showed higher sensitivity. Two serum and three bone marrow samples remained negative, although they were positive by the nested PCR assay. New serum samples were collected from the same subjects within a short time period, and were shown in a later study to to be positive for B19V genotype 2 (Cohen et al. 2006). These studies proved further the incapability of qPCR A to detect B19V genotype 2.

In the Netherlands a large number of plasma donations have been screened for B19V DNA by combining qPCR A and in-house qPCR assay capable of detecting all B19V genotypes (Koppelman et al. 2004, 2007, and 2011). Between 2006 and 2009, 6.2 million plasma donations were screened, from which 363 containing high B19V DNA titers. These samples were analyzed further (Koppelman et al. 2011). Fifteen out of 363 samples were negative in qPCR A but positive in in-house qPCR assay. The B19V strains in these samples were sequenced, and 3 samples with B19V genotype 2 were detected. The ability of qPCR A to detect different B19V genotype 1 isolates was also poorer than that of the in-house PCR. As well as primers and probes of qPCR A are incapable to detect B19V genotype 2, those are prone to miss isolates of B19V genotype 1.

In summary, the results obtained here (Study I), and in literature, qPCR B allows the detection, quantification, and differentiation of the B19V genotypes. As a real-time qPCR, it is rapid, less laborious, and time saving when compared to qualitative PCR assays neccesiating agarose gel electrophoresis. The qPCR assay B can be recommended for screening of plasma pools for B19V DNA.

B19V genotypes in blood (I and II)

Number of studies has described B19V genotype 1 DNA in medical blood derived products used for transfusion purposes. Prior to the beginning of this thesis, circulation in Europe of B19V genotype 1 but not genotypes 2 or 3 was reported in several studies. To examine the prevalence of B19V genotypes 2 and 3 in blood samples, 292 randomly chosen blood donor maxipools, consisting of 140 160 individual plasma samples, and 11 highly B19V viremic single blood donations, were analyzed (Study I). In addition, 1640 sera collected from patients with rash, or various other virus infection-related symptoms, including subjects with serologically confirmed *erythema infectiosum*, were examined for B19V genotypes (Study II).

The maxipools had been pre-screened by qPCR A at the Blood Service unit of the Finnish Red Cross. In this thesis, the maxipools were screened by qPCR B and the results were further confirmed by sensitive, qualitative VP1-PCR and K71-PCR (I). The majority of samples, 225 of 292, were negative with both qPCR assays. The samples that were positive can be divided into three different groups. Group 1 consisted of 13 samples positive for B19V DNA by both qPCR A and qPCR B; group 2 consisted of 3 samples qPCR B positive but qPCR A negative; and group 3 consisted of 51 samples which were positive by qPCR A but negative by qPCR B.

For verification, the samples found positive by qPCR B were examined by VP1-PCR and K71-PCR. All the samples analyzed by K71-PCR, which recognizes only B19V genotype 2, were negative. Out of 13 samples in group 1, seven were positive by VP1-PCR and contained B19V DNA $\geq 10^4$ IU/ml, as quantified by qPCR A. The six negative samples contained B19V DNA less than 10^3 IU/ml. The melting curve analysis after qPCR B amplification showed peaks corresponding to genotype 1 for the majority of samples. However, five samples gave melting peaks at approximately 63.6°C. This temperature is intermediate between the melting points characteristic for genotypes 2 and 3. No melting point was observed for one qPCR B-positive sample. The 51 samples in group 3 were low in B19V DNA quantity, less than 10^3IU/ml in 50 samples and 10^3-10^4 IU/ml in one. All samples had been screened by qPCR A soon after collection, and stored frozen at -20°C (with two thawings) before qPCR B testing.

Eleven single blood donations containing high titers of B19V DNA were analyzed by qPCR B for B19V genotypes (I). All of the donations were found positive for B19V DNA by qPCR B. The DNA extractions of these samples were done by both phenol-chloroform extraction and by the QIAamp DNA Mini Kit and the DNAs purified by the commercial kit showed slightly higher DNA concentrations than by the traditional way

with phenol-chloroform. The melting points of the phenol-chloroform extracted DNAs were unusually low, mean 64.9°C. When these samples were diluted 1:100 or DNAs extracted by QIAamp DNA Mini Kit, all except one, showed a melting peak characteristic for genotype 1. The melting peak for the one plasma donation (number 7) was approximately 63°C, again intermediate between genotypes 2 and 3. The amplicon generated from plasma donation number 7 was sequenced and was shown to contain B19V genotype 1 with some nucleotide changes. In conclusion, all 11 single blood donations contained DNA of B19V genotype 1 (I).

Around 1640 serum samples, collected between 1983 and 1997 from subjects with various virus infection- related symptoms, were examined as pools of ten for B19V DNA by VP1-PCR, genotype 2-specific K71-PCR, and by qPCR B (Study II). B19V DNA was found in 17% of pools. According to melting curve analysis and genotype 2-specific PCR, all the B19V positive samples contained B19V genotype 1 DNA. Genotypes 2 and 3 were absent from all the sera.

In conclusion, the results obtained from 140 160 blood donations studied in maxipools (I), and from 1640 serum samples (II), viremic infections by B19V genotypes 2 and 3 are extremely rare in Northern Europe. This view is largely supported by other studies, which further widen the geographical area from Northern Europe to include all of Europe, and in case of genotype 2 to the entire globe. In two studies Heegaard et al. (2001, 2002a) examined 100 000 blood donor plasma samples, 100 individual sera containing B19V IgM, and 190 bone marrow samples from healthy subjects for B19V genotypes 1 and 3. In both studies, B19V genotype 1 was the only genotype found. Servant et al. (2002) evaluated the prevalence of B19V genotypes in France in 1084 clinical samples. B19V DNA was detected in 396 samples, of which 385 represented genotype 1. Genotype 2 DNA was found in two samples, and genotype 3 in 9 samples. Both V9 and D91.1 isolates of genotype 3 were represented. In the USA, Nguyen et al. (2002) examined 149 sera and 18 bone marrow samples for B19V DNA, and found 29 sera to contain B19V DNA. Only one sample contained genotype 2. Among 1411 clinical samples sent for B19V diagnosis in the UK, the majority contained B19V genotype 1 DNA (Cohen et al. 2006). Genotypes 2 and 3 were detected only in one (separate) case.

At the beginning of this thesis in 2003 two new B19V genotypes were identified. However, most published and commercially available PCR methods for B19V DNA detection were designed by using sequences of B19V genotype 1 and thus missed the detection of B19V genotypes 2 and 3. After that, several PCR methods have been evaluated for genotypes 2 and 3, and new detection methods have been established. With the advent of current methods for the detection of B19V genotypes 2 and 3 it has

been possible to make a notion that the occurrence of B19V genotype 2 is, indeed, sporadic, and not due the unsuitable detection assays. In the Netherlands between the years 2005 and 2009 approximately 8.8 million plasma donations were screened by qPCR for high titer B19V plasma. According to sequencing data, only four plasma donations contained B19V genotype 2. Genotype 3 was not detected in any of the donations screened (Koppelman et al. 2007, Koppelman et al. 2011). Medical plasma derivatives were also examined for B19V in South Korea and among more than 10 000 donors, none represented B19V genotypes 2 and 3, but genotype 1 was present (Oh et al. 2011). Genotype 2 has also been found in the serum of a pregnant woman (Enders et al. 2006), in a donated plasma sample (Blumel et al. 2005), in blood of a transplant recipient (Liefeldt et al. 2005) and in blood of the two kidney-transplant recipients (Grabarczyk et al. 2010).

Endemic distribution of B19V genotype 3 is mainly restricted to African and South-American areas(Table 6) (Candotti et al. 2004, Candotti et al. 2006, Parsyan et al. 2006, Sanabani et al. 2006, Freitas et al. 2008). Candotti et al. (2004, 2006) found 1.3 % of Ghanaian blood donors to be positive for B19V genotype 3 DNA, and later, B19V-containing sera from pregnant women were mostly positive for genotype 3 DNA. Parsyan et al. (2006) studied the sera of 200 Ghanaian children and reported a B19V DNA prevalence of 11.5%. All of these samples contained B19V genotype 3. The sera were also analyzed for B19V antibodies by ELISA, and 34.8% were positive for IgM antibodies suggestive of acute B19V infections (Parsyan et al. 2006). Furthermore, B19V genotype 3 has been detected among patients with B19V-related symptoms in the Amazonian region in Brazil with a prevalence of 9% (Freitas et al. 2008).

Results of Studies I and II, and others, strongly indicate that genotype 2 has disappeared from wide circulation In addition, B19V genotype 3 was absent in our serum and plasma samples but has been detected endemically elsewhere. Table 6 summarizes the prevalences of B19V genotypes detected in blood.

***Table 6**. Different distribution of B19V genotypes 2 and 3 detected in blood samples.*

Study	Country	N=	Genotype 2	Genotype 3
Nguyen et al. 1999	France	1	ns*	1
Heegaard et al. 2001	Denmark	100 100	ns*	0
Heegaard et al. 2002	Denmark	190	ns*	0
Nguyen et al. 2002	USA, Denmark	287	1	0
Servant et al. 2002	France	1084	2	9
Candotti et al. 2004	Sub-Saharan Africa, UK	2440	0	12, Ghana
Schneider et al. 2004	Germany	202	5	ns*
Blümel et al. 2005	Germany	6	1	0
Liefeldt et al. 2005	Germany	1	1	ns*
Cohen et al. 2006	UK	1411	1	1
Candotti et al. 2006	Ghana	885	0	14
Enders et al. 2006	Germany	185	1	0
Parsyan et al. 2006	Brazil	200	ns*	23
Sanabani et al. 2006	Brazil	69	1	6
Koppelman et al. 2007	Netherlands	2.6 million	1	0
Freitas et al. 2008	Brazil	487	0	11

Study	Country	N=	Genotype 2	Genotype 3
Hübschen et al. 2009	Kyrgyzstan, Bulgaria, Burgina Faso, Estonia, Georgia, Greece, Israel, Luxembourgh, Nigera, Russia, Serbia	152	0	35, Nigeria, Burkina Faso,Greece, Bulgaria, Kyrgyzstan
Keller et al. 2009	Brazil	842	0	1
Rinckel et al. 2009	USA	810 000	0	1
Grabarczyk et al. 2010	Poland	69	2	0
Koppelman et al. 2011	Netherlands	6.2 million	3	0

*ns indicates not studied

Parvovirus B19V persistence in solid tissues (II and V)

After primary infection, B19V genomic DNA remains detectable in human tissues in both symptomatic and asymptomatic subjects. In Study II, the extent of the DNA persistence of the different B19V genotypes was determined by examining 523 tissues of several types by qualitative and quantitative PCR assays. In addition, 100 biopsies of heart tissue, collected from adult patients undergoing open-heart surgery in Germany, were examined for B19V DNA (Study V).

Altogether 190 (36.3%) samples of 523 dermal, synovial, tonsillar and liver tissues contained parvovirus B19V DNA (Table 7). B19V genotype 1 was found in 132 (25%) tissues and B19V genotype 2 in 58 (11%) tissues. After amplification by qPCR B, the melting curve analysis in the case of two tonsillar tissues showed a melting point of 63°C. Similar to the case of plasma samples in Study I, after sequencing, the B19V genomes in the two tonsils turned out to be subvariants of genotype 1. B19V genotype 3 was absent from all the tissues examined (Table 7). Importantly, all the tissue donors carrying B19V DNA were seropositive for B19V IgG.

In the study of German heart biopsies (Study V), B19V DNA was found in 85 (85%) heart samples. Genotype 1 was detected in 9 (9%) and B19V genotype 2 DNA in 76 (76%) biopsies of heart. Genotype 3 was absent also from these tissues.

Both genotypes, 1 and 2, were found in all tissue types studied. Thus, the universal distribution of both B19V genotypes ruled out tissue type specificity of any of the B19V genotypes. In addition, our results point out that B19V DNA persistence is a characteristic for this virus among healthy subjects.

***Table 7**. Number of tissues studied for the DNA of B19V genotypes and the prevalences obtained.*

Tissue	Genotype (%)			Negative	Total
	1	2	3		
Skin	41 (31%)	24 (17%)	ND*	73 (52%)	140
Synovia	30 (35%)	7 (8%)	ND	49 (57%)	86
Tonsil	36 (16%)	3 (1%)	0 (0%)	181 (82%)	220
Liver	25 (32%)	24 (31%)	ND	28 (36%)	77
Heart	9 (9%)	76 (76%)	0 (0%)	15 (15%)	100

*ND, not done

In human solid tissues, B19V DNA prevalence rates similar to Study II, have been shown among symptomatic and asymptomatic tissue donors (Söderlund et al. 1997, Eis-Hübinger et al. 1999, Wong et al. 2003, Manning et al. 2007, Corcioli et al. 2008, Bonvicini et al. 2010, Schenk et al. 2009, Ruppert et al. 2011). Both B19V genotypes 1 and 2 have been detected in different tissue types including brain, skin, bone marrow, spleen, heart, and liver (Manning et al. 2007, Schneider et al. 2008a, Schenk et al. 2009, Zakrewska et al. 2009, Bonvicini et al. 2010). B19V genotype 3 have been found in tissues in only few cases, with low prevalence rates; between 2.2% and 3.4% in livers, 2.6% in synovium, and between 1.4% and 5.2 % in the heart (Wong et al. 2003, Corcioli et al. 2008, Schneider et al. 2008a, Ruppert et al. 2011). However, genotype 3 has been detected in 23% of BM samples collected in Brazil from subjects with past serologically-verified B19V infection (Garcia et al. 2011).

Distribution of B19V genotypes (II)

Tissue donors in Study II were grouped according to the birth year, and they were analyzed for the prevalence of B19V genotypes (Figure 1 in II). B19V genotype 1 was seen almost uniformly in subjects of all ages. Among those born in 1950s or earlier the prevalences for genotype 1 and 2 were similar, 22% and 28%, respectively. In contrast, genotype 2 occurred in merely 3% in the individuals born in the 1960s, and in only a single individual born in the 1970s. Overall B19V DNA prevalence was low (4%) in children, and uniformly of genotype 1. None of the 1640 sera, collected in the 1980s and the 1990s (II), contained B19V genotype 2.

Studies II and V presented similar tissue donor age distributions; the prevalence of B19V genotype 2 increased in older age groups. Among heart biopsy donors (Study V) aged less than 60 years, 25% were positive for genotype 1 and 75% for genotype 2. Among those, aged from 60 to 69 years, 8% were positive for genotype 1 and 92% for genotype 2. Among those, aged from 70 to 79 years, the prevalences for genotype 1 and 2 were 11% and 89%, respectively. All patients aged over 80 years carried solely B19V genotype 2 DNA.

The findings of the two B19V genotypes in human tissues suggested that there are are genotypic variations in the viruses that are in circulation in the population. It was found that the newly-found variant, genotype 2, is in fact older in occurrence than the prototypic genotype 1. Subjects with persistent infections by genotype 2 were born on the average 20 years earlier than those carrying genotype 1. Therefore, assuming for genotype 2 preferential acquisition during childhood and adolescence, as holds for genotype 1, both genotypes have circulated in Northern and Central Europe in equal frequencies since the 1930s to 1950s. However, genotype 2 appears to have disappeared from wide circulation by the 1970s and has remained absent ever since. This result is further supported by the results with serum samples, as well as by the sporadic genotype 2 cases in blood discussed in the chapter 'B19V genotypes in blood'. Results reported in Scottish and German studies are in line with this study. In a B19V genotype prevalence study, 68% of liver tissues contained B19V DNA, and the birth year ranges for genotypes 1, 2, and 3 positive subjects were 1923-81, 1916-51, and 1939-74, respectively (Schneider et al. 2008a). In two other studies of B19V DNA in heart biopsies, a clear age distribution was also seen between those who carried genotypes 1 and 2; genotype 1 was detected among those born between 1950 and 1969 and genotype 2 among those born between 1927 and 1957 (Schenk et al. 2009). In a study by Kühl et al. (2008) the B19V genotype 1 was preferentially found in the

heart biopsies of patients aged below 45 years, whereas patients above 45 years carried mostly genotype 2, and those over 65 years carried only genotype 2. In a study by Peter Simmond's group, the subjects carrying genotype 1 were born approximately 10 years later than those carrying genotype 2 (Manning et al. 2007).

The results with tissue and sera in Studies I and II, as well as those in the literature, do indicate different circulation of B19V genotypes; B19V genotype 2 was circulated in Northern and Central Europe several decades ago and then disappeared from wide circulation. Furthermore, B19V genotype 3 has not circulated widely in Europe during the past 70 years, even if it is endemic in South-America and Africa. Today the most prevalent circulating virus type in Europe is genotype 1.

The reasons for these epidemiological differences between three B19V genotypes are not known. Promoters and NS1 proteins of the three genotypes have been shown to be equally active (Ekman et al. 2007, Chen et al. 2009). Furthermore, in the coding area of structural proteins, the variation between genotypes is at its lowest level; at the DNA level ~10% and at the amino acid level less than 2% (Hokynar et al. 2002). These variations in capsid structure have not been observed to affect the infectivity of B19V genotype 2 in perimissive cell lines (Blümel et al. 2005, Ekman et al. 2007). These data suggest that sequence variations have not reduced the fitness of genotype 2. However, changes in the use of the first splicing acceptor, between genotype 1 and 2, have been detected, as well as changes in replication of genotype 2 and 3 genomes cloned in between of ITRs of genotype 1 infectious clone. Whether these latter changes could have affected the circulation of B19V genotype 2, remains to be determined. An entirely different explanation for the occurrence of B19V genotype 2 among the elderly could be their selective susceptibility to primary infections of this genotype. However, such an inverse chronological tropism is highly unlikely because it would imply continued circulation of genotype 2, which was ruled out by the results presented in studies I and II, and this has not been shown for any human virus known.

Collectively, the data reported here indicate that human tissues possess, regarding the genomes of single-stranded DNA viruses, a storage mechanism of lifelong capacity and provides a registry of individual´s encounters with infectious agents. At the epidemiological level this lifelong persistence provides a database for the analysis of the occurrence and circulation of viruses and their variants. This concept was designated as the *Bioportfolio*.

B19V and heart diseases (V)

B19V has been frequently found in tissues of symptomatic and asymptomatic adults, including heart biopsies of subjects with and without symptoms of myocarditis or cardiomyopathy, but it is not fully known whether B19V is related to ethiology of cardiac diseases. In this study, B19V DNA prevalence in heart tissue obtained from patients who had myocardial diseases with unknown ethiology was analyzed.

Left atrium tissues of 100 patients undergoing open heart surgery, including valve replacement or correction, and coronary artery bypass grafting, were collected and examined by quantitative PCR for B19V DNAs. In B19V DNA positive tissues, the genotyping was done by qualitative PCR assays. In addition, the patient's sera were analyzed for B19V antibodies by EIA and immunoblotting. B19V DNA was detected in 85% of tissues and both genotypes 1 and 2 were found. The mean quantity of B19V DNA detected was 146.6 GE/10^6 cells, and the maximum was 1025 GE/10^6 cells. The ejection fraction was >55% in 58 patients and < 35% in 16 patients. The quantity of B19V DNA was not significantly different between the patients with different ejection fractions. Most of (96%) patients were B19V IgG seropositive.

The B19V copy numbers detected in heart tissue are similar to those in human skin, synovial or tonsils reported in Study II. In diseases, associated with B19V acute infection, copy numbers are significantly higher. It has been reported that B19V may induce acute myocarditis that clinically mimics ischemic heart disease, and that high quantity ($4.3x10^5$ copies/μg) of B19V DNA was detected in endomyocardial biopsy obtained from 34 years old woman, who encountered fatal B19V infection (Bultman et al. 2003). Bock et al. (2010) have also reported the different B19V quantities in inflamed and non-inflamed hearts, and suggested that B19V copies above 500GE/μg DNA would be clinically relevant threshold for myocardial inflammation. Of note, B19V DNA quantities 10^1-10^5 GE/μg DNA in heart tissues, have been observed in samples from subjects with and without inflammation (Schenk et al. 2009, Lindner et al. 2009, Steward et al. 2011).

Inflamed tissues are infiltrated by leukocytes such as natural killers and macrophages as well as proinflammatory cytokines (Feldman et al. 2000). Lindner et al. (2009) investigated whether adaptive immune responses, among patients with acute myocarditis or dilated cardiomyopathy, and prevalences of B19V DNA in their heart tissue, are different from those observed in healthy individuals. No differences in the T-cell mediated immune responses were observed. The result suggested that the mere precence of B19V DNA in heart tissue is not of clinical significance.

In study by Kühl et al. (2008), the most prevalent B19V genotype in heart of patients with dilated cardiomyopathy was genotype 2. The patients with B19V genotype 1 were younger and had poorer left ventricular function than those with genotype 2. Thus, genotype 1 was proposed to trigger the more severe cardiac dysfunction than genotype 2. In Study V, genotype 1 was found among older and younger patients, as well as in the study with other tissue types (Study II). B19V genotypes have been shown to be equally active, infect the same cell types, and represent one serotype (Blümel et al. 2005, Ekman et al. 2007). Thus far, clear cut evidence of different pathobiology of B19V genotypes has not been reported. Even if B19V primary infection may lead to acute cardiac disease, the results in this study (V) and in others indicate that the B19V DNA in heart tissue of seropositive patients is not predictive for the outcome of myocarditis or cardiac myopathy.

Newly found human parvoviruses in human tissues (IV, V)

The prevalence of human parvovirus 4 and human bocaviruses in tonsillar, dermal, and synovial tissues was determined in order to answer the question of whether tissue persistence is a universal property of parvoviruses (Study IV). Prior to PCR analysis for viral DNA, the success of DNA extraction and the quality of the DNA after long storage was ensured by PCR for human β-globin gene. All of the tissues gave positive β-globin PCR results. In addition, the heart biopsies from German subjects were studied for HBoV1 DNA (Study V).

Prevalence of human parvovirus 4 in tissues and blood (IV)

Altogether 334 solid tissues were examined by nested PCR for PARV4 DNA. The sensitivity of the PARV4 PCR was determined with ten-fold dilutions of a PARV4 containing plasmid, and a sensitivity of 10 copies/reaction was observed. Tissues of tonsils (N=215), synovia (N=56), and skin (N=63), contained no PARV4 DNA. The PARV4 plasmid used as a positive control in each PCR run, gave a positive results in agarose gel electrophoresis, indicating the successful performance of PARV4 PCR.

The presence of PARV4 VP2 antibodies were also examined in serum samples obtained from a subset (N=199) of the tonsillae from child and adult donors by IgM and IgG EIAs. No samples contained IgM or IgG against PARV4, while positive samples from a study by Lahtinen et al. (2011) served as controls.

In the literature, PARV 4 has been detected in tissue and blood samples (Table 8). Viremia seems to be the hallmark of PARV4 infection. PARV4 was found in 16% of Clotting factor VIII concentrates with a higher prevalence in older products, manufactured 30-35 years ago (Fryer et al. 2007b). Similar results were obtained by Schneider et al. (2008a) who reported a prevalence of 33% in older coagulation factor concentrates and a prevalence of 9% in the most recently manufactured concentrates. In a study from Thailand, the PARV4 prevalence among IDUs was higher (7.95%) than among blood donors (3.95%).

Information of blood donors is rarely available from blood-derived medical products. However, Fryer et al. (2007b) described the source of the blood in one of the plasma products manufactured in year 2000. This product consisted of blood collected from paid donors (Fryer et al. 2007b). Plasma products like these may have originated from IDUs or subjects, who are HIV- or HCV infected. The higher prevalence of PARV4 DNA in older coagulation factor concentrates reflects, most probably, the situation when instructions for blood safety were not as strict as today. After mid 1980s the instructions have changed in response to the HIV epidemics, and plasmas containing HCV or HIV have been destroyed.

PARV4 has been found mainly in lymphoid tissue and bone marrow of HIV-infected IDUs (Longhi et al. 2007, Manning et al. 2007, Simmonds et al. 2007), but also in other tisssues (Schneider et al. 2008b, Botto et al. 2009, Corcioli et al. 2010) (Table 8). This may be due to the fact that population exposure to PARV4 is more restricted than to B19V. The published PARV4 DNA findings in tissues and blood suggest that the transmission route for PARV4 is parenteral, at least for PARV4 genotypes 1 and 2. This is supported by results of serological studies in which PARV4 IgG is detected with prevalences of 67% and 78% among IDUs, but 0% among healthy controls (Sharp et al. 2009, Lahtinen et al. 2011). PARV4 antibodies were not detected among healthy blood donors from France, or among the general population in the United Kingdom (Sharp et al. 2010). Parenteral exposure is furthermore supported by a recent study of PARV4 serology, which describes the transmission of PARV4 by virally inactivated clotting factor concentrates (Sharp et al. 2011). This study also shows that PARV4 is resistant, at least, to some viral inactivation procedures.

PARV4 has three genotypes, which interestingly have similar age and geographical distributions to B19V genotypes. PARV4 genotype 2 has been found mainly in older tissue donors than genotype 1 (Manning et al. 2007, Schneider et al. 2008b). Furthermore, PARV4 genotype 2 is more prevalent in older plasma pools and coagulation factor concentrates compared to genotype 1, for which prevalence is

higher in the most recent blood products (Fryer et al. 2007b, Schneider et al. 2008a). PARV4 genotype 3 has been found only in sub-Saharan areas where it also occurs among healthy subjects and infants (Simmonds et al. 2008, Panning et al. 2010, Sharp et al. 2010). The reasons for these epidemiological differences are not known.

Taken together, the results presented in Stydy IV and results by others indicate that PARV4 genotypes 1 and 2 are parenterally transmitted. The result that no PARV4 DNA was found in tissues collected from children and adults without history of drug use, HIV or HCV is thus not surprising, reflecting the absence of exposure to this virus in Northern Europe. The absence of PARV4 IgG and IgM further confirmed this conclusion.

Table 8. *PARV4 DNA findings by PCR.*

Study	Sample material	N=	PARV4 DNA (%)	PARV4 DNA+ sample material
Jones et al. 2005	plasma	25	1 (4%)	IDU, HBV
Fryer et al. 2006	plasma pools	137	7 (5.1%)	-
Fryer et al. 2007a	plasma pools, individual plasma	766 416	67(8.7%) 17 (4%)	healthy donors, plasma pools collected between 1990-1993, febrile patients, including IDUs
Fryer et al. 2007b	VIII concentrates	175	28 (16%)	Collected in the 1970s and 1980s
Fryer et al. 2007c	blood	26	3 (11.5%)	HCV and/or IDU
Manning et al. 2007	bone marrow brain lymphoid tissue	31 29 31	15 (48%) 0 (0%) 14 (45%)	HIV, IDU, hemophilia
Longhi et al. 2007	bone marrow	35	16 (40%)	HIV, IDU
Simmonds et al. 2007	lymphoid tissue, bone marrow	51 51	14 (27%) 14 (27%)	IDU, HIV
Lurcharchaiwong et al. 2008	serum	264	14 (5.3%)	IDU + blood donors negative for HIV, HCV and HBV

Study	Sample material	N=	PARV4 DNA (%)	PARV4 DNA+ sample material
Schneider et al. 2008a	coagulation factor concentrates	169	21 (12.4)	Coagulation factor concentrates from 1980s, 1997 and between 2000 and 2003
Schneider et al. 2008b	liver	87	13 (15%)	HCV infection, alcoholic liver cirrhosis, liver failure of unknown reason
Simmonds et al. 2008	lymphoid tissue	13	7 (53%)	HIV, study subjects from sub-Saharan Africa
	bone marrow	13	6 (46%)	
Botto et al. 2009	blood	53	2 (3.8%)	healthy blood and skin donors, skin donors with dermatological disease
	skin	142	26 (18%)	
	broncho alveolar lavage	39	0 (0%)	
Vallerini et al. 2008	plasma, serum, PBMC	417	3 (0.6%)	2 kidney transplant recipients and 1 patient with suspected viral disease
Tuke et al. 2010	blood	460	8 (1.7%)	HCV, HIV, HBV

Study	Sample material	N=	PARV4 DNA (%)	PARV4 DNA+ sample material
Corcioli et al. 2010	bone marrow	26	2 (5.5%)	17 autopsy samples from subjects with various causes of death, subjects without known acute viral infections, immunodepressed hematological patients
	skin	25	1 (4%)	
	myocardium	35	17 (49%)	
	lung	17	4 (23%)	
	kidney	17	3 (18%)	
	liver	17	7 (41%)	
	synovium	21	1 (5%)	
	plasma	39	8 (20.5%)	
Panning et al. 2010	blood	279	24 (8.6%)	Ghanaian infants
Benjamin et al. 2011	cerebrospinal fluid	12	2 (16.7%)	central nervous system infection
Chen et al. 2011	blood	4	3 (75%))	mothers and infants with hydrops
Touinssi et al. 2011	plasma	289	71 (24.5%)	hemodialysis patients with or without HBV markers, lung transplant recipients

Occurrence of human bocaviruses and corresponding antibodies against them (IV, V)

Altogether 557 biopsies of tonsils, skin, and synovium were examined for HBoV1 DNA by qualitative or quantitative PCR (Study IV). Biopsies of skin and synovium were negative, while 16 (3.4%) tonsillar tissues contained HBoV1 DNA (Table 9). The quantity of HBoV1 DNA was low (≤ 461 copies/mg tissue). All tonsils positive for HBoV1 DNA were collected from young children, 2 to 8 years of age.

Serum samples from 496 tissue donors were studied for HBoV1 antibodies (Study IV). The IgG prevalence rate of 94.9% was obtained for the tonsil donors and 88% for those who donated skin and synovia samples. No HBoV1 IgM was detected among any of the donors. Those 16 children, who carried HBoV1 DNA in their tonsils, were HBoV1 IgG positive by regular EIA and fifteen sera of them were further assayed by VLP competition, after which eleven (73.3%) exhibited HBoV1-specific antibodies. Combined HBoV2/3 antibodies were detected in six (40%) subjects. One sample did not show reactivity against any of the HBoVs when assayed after VLP competition. No HBoV1-4 DNA was detected by by multiplex qPCR in the serum of the HBoV1 DNA-positive children.

A subset of tonsillar tissues (N=215), available after HBoV1 analysis, was examined by HBoV1-4 multiplex PCR for HBoV2-4 DNAs (Study IV). No positive PCR results were observed for HBoV1-4. Corresponding serum samples from most of the tonsillar tissue donors (N=212) were studied for HBoV1-3 antibodies by VLP competition assay. After removing cross-reacting HBoV2/3 IgG antibodies by VLP competition the prevalence solely for HBoV1 IgG was 68.4%. After competition with HBoV1 VLPs the combined prevalence for HBoV2/3 was 28.3%.

In addition to the tissues in Study IV, 100 biopsies of heart collected during open heart surgery from patients without cardiomyopathy or myocarditis had been examined for HBoV1 DNA in Germany (Study V). HBoV1 DNA was detected in 5 heart biopsies, in all cases from adults. All of these HBoV1 DNA positive subjects carried HBoV1 IgG in their serum.

***Table 9**. Geno- and seroprevalences of HBoV1 by PCR and regular EIA.*

HBoV1	Tissue donors (N=657)			
	tonsils (N=438)	synovia (N=56)	skin (N=63)	heart (N=100)
DNA prevalence, %	3.4%	0%	0%	5%
Seroprevalence, %	94.9%	87.5%	85.7%	96%

The receptors, host cells, and the entire cell biology of HBoV1-4 are unknown. Nevertheless, one study reported the replication and transcription activity of HBoV1 in pseudostratified human airway epithelium (Dijkman et al. 2009). Tonsillar tissues, examined in large numbers in Study IV, represent both upper airway epithelium and lymphoid tissue, and could be the tissue of HBoV1 persistence if such occurs. We found HBoV1 DNA in the tonsils of 3.7% subjects, all of whom were children. In contrast, the skin and synovia that were from adults, as well as the tonsils of adults, harbored no HBoV1 DNA. All 16 HBoV1 DNA-positive tonsillar donors were also HBoV1 seropositive by the regular EIA, while 4 became negative for HBoV1-specific IgG after VLP competition. This seronegativity does, however, not necessarily mean that these children could not have been exposed to HBoV1 in their past. Competition assays, by definition, eliminate cross-reactive antibodies, including antibodies towards common epitopes, and therefore initially low IgG titer could lead to a "false" negative competition-EIA result (Kantola et al. 2011).

The presence of HBoV1 IgG and the absence in the serum of both HBoV1 IgM and HBoV1 DNA indicates that the children in this study (IV) did not have an ongoing acute HBoV1 infection, but rather had been infected in the past. In prior studies HBoV1 DNA in tonsils or adenoids with prevalences of 5.5% and 32% were described (Table 10) (Lu et al. 2008, Clément et al. 2009). However, in those studies neither HBoV1 PCR in serum nor serology, were conducted, thus it is difficult to determine whether the described HBoV1 DNA prevalences were due to acute HBoV1 infection or remained from past infection. HBoV1 DNA has been shown to remain detectable for months in the respiratory tract after acute infection (Brieu et al. 2008, von Linstow et al. 2008, Martin et al. 2010, Lehtoranta et al. 2012) and has been found in nasopharyngeal aspirates of 43% asymptomatic children undergoing elective surgery (Longtin et al.

2008). Whether the findings of past-infection DNA positivities made in Study IV represent prolonged replication and shedding, passive persistence after primary infection, or mucosal (*in vivo*) contamination, remains to be shown.

In contrast with the results obtained from adult synovium, tonsil, and skin donors, 5% of heart biopsies contained HBoV1 DNA. All of the HBoV1 DNA-positive heart donors were HBoV IgG positive and IgM negative (Study V), showing no evidence of HBoV1 acute infection. In samples from adults, HBoV1 DNA was reported in 18% of paranasal sinuses or nasal polyps in patients undergoing elective surgery for chronic sinusitis (Falcone et al. 2011). Whether these HBoV1-persistence data have any connection with disease pathogenesis remains to be determined.

***Table 10**. HBoV1-3 DNA in human tissues.*

Study	Tissue	N=	HBoV1-4 DNA (%)	Children/adults
Lu et al. 2008	tonsils, adenoids	164	*HBoV1*: 53 (32.3%)	children
Clément et al. 2009	tonsils, adenoids	91	*HBoV1*: 5 (5.5%)	children
Falcone et al. 2011	paranasal mucosal tissue, nasal polyps	102	*HBoV1*: 18 (17.6%)	adults
Kapoor et al. 2011	intestine	22	*HBoV2*: 1 (4.5%) *HBoV3*: 3 (13.6%)	children

The age distribution of tonsillectomy patients in Study IV was wide, ranging from 1.5 to 72 years. Among children ≤ 8 years of age, the HBoV1 DNA prevalence was 9.5%. In addition, more than half of the patients were HBoV1 seropositive. Skin and synovium donors were adults with a high HBoV IgG seroprevalence rate. Nevertheless they did not harbor HBoV1 DNA in their tissues. Among the heart biopsy donors, the HBoV1 seroprevalence rate was as high as 96%, yet only a few of them carried HBoV1 DNA in tissues. In contrast to this, B19V DNA was detected in 48% of synovial, in 43% of skin,

and in 85% of heart biopsies of the B19V seropositive adult subjects (Studies II, V). Our HBoV PCR results indicate that HBoV1 can remain detectable in tonsillar tissue of children, long after primary infection, and may randomly occur in coronary specimens, yet without lifelong persistence.

The tonsillar biopsies lacked HBoV2-4 DNA (IV). Even though only 28% of the children were seropositive for HBoV2/3 combined, indicating past infection of HBoV2/3, it may be that IgG is not a good marker of past exposure to these enteric viruses as the infections may not be systemic. HBoV2 and 3 have been found in small numbers (Table 7) of intestinal biopsies (Kapoor et al. 2011), which could be the tissue type for future studies of HBoV2-4 persistence.

To summarize, PCR and serological data does not support the possibility of lifelong persistence of HBoV1 genomes in solid tissues, but instead point to slow evanescence from tonsillar tissues in young children after primary infection. The mechanism of HBoV1 DNA occurrence in tonsils remains to be clarified.

Possible mechanisms of tissue persistence of parvoviruses

It is not known which cell types support the persistence of B19V and PARV4 DNAs or the occurrence of HBoV1 DNA in human solid tissues. The receptors and host cells of PARV4 and HBoV1-4 are unknown. The primary receptor for B19V, P antigen, and other glycosphingolipids capable of binding B19V, have also been detected in cells other than erythrocytes. B19V has been shown to slowly replicate in the monocytic cell line U937 (Munakata et al. 2006). B19V vector, carrying marker genes, has been described to enter several epithelial cell lines (human umbilical vein endothelial cells, HUVEC, human embryonal kidney cells 293 and HeLa), and express the vector markers (Weigel-Kelley et al. 2001, Weigel-Kelley et al. 2003). In addition, the production of B19V NS1 in hepatocytes and the presence of mRNAs for NS1 and VP1 in dermal fibroblasts have been reported (Poole et al. 2004, Zakrewska et al. 2005). These data suggest that B19V entry into, and restricted expression, in cells other than erythroid, is possible.

The cellular and molecular mechanisms of the tissue persistence of B19V (and PARV4) are unknown. Possible alternatives for viral persistence are: i) viral integration (known to occur with AAV-2, which is parvovirus); ii) endosomal persistence (reported for rat parvovirus), and iii) episomal storage (recently described for HBoV1 and HBoV3 and AAV). In addition, B19V as well as HBoVs and PARV4 could even be encapsidated and remain attached as full virions on the cell surface. The concept behind the mechanism

of the *Bioportfolio* could also involve equilibrium between viral replication and efficiency of the host immunity.

Several percentages of the human genome comprise endogenous viral elements. Most of these are derived from retroviruses, which normally integrate into the human chromosomal DNA. The endogenization occurs when the viral genome integrates into the host genome of reproductive germ- line cells. Endogenous occurrence of parvovirus genomes in different mammalian species has been described in two studies (Kapoor et al. 2010b, Belyi et al. 2010). The sequence of the structural protein gene of canine parvovirus (CPV) was used as a template in the screening of genetically related sequences in the genomes of several vertebrate (Kapoor et al. 2010b). Several sequences, homologous to exogenous parvoviruses, were found within the genomes of several mammals, including wallabies, opossums, guinea pigs, and hedgehogs. When all representatives of known exogenous parvoviruses were compared with the obtained endogenous sequences, two separate clades were observed. One was more similar to the genus *Parvovirus* and the other to genus *Dependovirus.* These results speak on behalf of integration of several parvoviruses. Belyi et al. (2010) described results in line with those of Kapoor et al. (2010b) for both parvoviruses and circoviruses. Kapoor et al. (2010b) also suggested that integration is characteristic not only for dependoviruses, which are known to integrate, but also for autonomous parvoviruses. Interestingly, endogenous sequences homologous to autonomous primate parvoviruses or human parvovirus B19 were not observed.

Endogenous occurrence of B19V DNA could explain the wide and long term persistence observed for B19V. However, if endogenous storage of B19V DNA would be the mechanism, it would be likely that the B19V DNA had commonly been amplified from subjects without history of B19V infection, meaning seronegative subjects. In the Studies II and V, in which large number of tissues were examined, this was not the case, but the B19V DNA-positive subjects were seropositive for B19V IgG. In other words B19V DNA was detected among those provably carried B19V infection in their past. B19V DNA has only been detected in tissues of seropositive subjects in other studies as well (Söderlund et al. 1997, Eis-Hübinget et al. 2001, Vuorinen et al. 2002, Shenck et al. 2009). In the study II, tissues obtained from young children rarely harbored B19V DNA, which does not support the endogenous mechanism for B19V persistence. In addition, the occurrence of HBoV1 in tonsillar tissue (IV) hardly represents endogenous persistence because most of the subjects were negative for HBoV1 DNA, and viral DNA was only observed in one tissue type. Furthermore, HBoV1 was found only in children. DNA persistence of PARV4 in lymphoid tissue among IDUs, is unlikely to be due to endogenous mechanism.

If endogenous parvoviral elements would exist in human chromosomal DNA it would be of interest if those were to be expressed along with host genes. If parvovirus-like proteins would appear on the cell surface together with host proteins, this could in theory lead to adaptation of the host immune system for parvovirus-like domains and, thus, support persistence, or low-level replication of B19V in permissive and non-permissive cells. In other words for example to prevent the regocnition of B19V infected cells by lymphocytes.

The life cycle of adeno-associated virus 2 (AAV-2) that belongs to the genus *Dependovirus,* comprises two phases. In the presence of helper virus AAV-2 replicates actively to levels of 10^5 virus particles per cell. In the absence of helper virus AAV establishes latency (Dutheil and Linden 2005). During latency AAV-2 genome integrates site-specifically into chromosome 19 (Kotin et al. 1990, Samulski et al. 1991) by a non-homologous recombination mechanism, and is mediated by AAV-2 nonstructural *rep* proteins (Surosky et al. 1997). Integration of B19V has not been described, but similar mechanisn as observed in AAV-2 could explain the long-term persistence of B19V in human tissues.

Another latency-related form of AAV-2 genome was detected in human tonsillar tissue in spleen and in lung tissues. In the study by Shnepp et al. (2005) site-specific integration of AAV-2 was not detected by PCR designed to amplify the viral-cellular DNA junctions. Instead of integrated AAV-2, double-stranded head-to-tail oriented circular AAV-2 episomes were detected. Furthermore, Penaud-Bodloo et al. (2008) showed that the recombinant AAV-2 vector injected into muscle of non-human primates also occurred as monomeric or concatemeric episomes, and that cellular histones were regularly positioned along the episomes creating a chromatin-like structure. One could hypothesize that this chromatin-like structure could maintain the tissue persistence of AAV-2 episomes and protect the virus from degradation by host cell machineries.

Recently, a head-to-tail oriented circular form of HBoV1 was reported in respiratory samples by a German group (Lüsebrink et al. 2011). The head-to-tail structure contained genomic sequence without any hairpin structures. The episomal structure was formed by covalent linkage of novel 54 base pair sequence, of which 20 base pairs are also detected in the palindromic ends of MCV (Lüsebrink et al. 2011). This novel HBoV1 structure of Lüsebrink et al. was confirmed by Kapoor et al. who detected HBoV3 head-to-tail sequences in intestinal tissues obtained from patients with diarrhea (Kapoor et al. 2011, Schildgen et al. 2012). Assays used in both of the latter studies could have detected integration of HBoV1 or HBoV3, but no integration was observed.

The results described for AAV and HBoVs illustrate the ability of different parvovirus genomes to occur as circular forms in solid tissues and respiratory specimens.

B19V episomes have not been described. The recombinant AAV-2 episome, in close contact of chromatin-like structures, could also be imagined to be the mechanism of B19V persistence. With HBoV1 and HBoV3, it is not clear, similar structures are mechanisms of persistence/long term occurrence or actually the replicative forms of human bocaviruses. The exact replication mechanism of human bocaviruses is not known and the specimens where episomes of HBoV1 and HBoV3 were found could represent the type for persistence as well as the type where those bocaviruses actively replicate during acute infection. However, the current replication model of parvoviruses does not support the hypothesis of replicating episomes, i.e. the rolling circle mechanism.

It is not known whether B19V can be activated from persistence, but genotypes 2 and 3 have been encountered in the blood and bone marrow of immunosuppressed subjects relatively often compared to immunocompetent individuals (Nguyen et al. 2002, Servant et al. 2002, Liefeldt et al. 2005, Cohen et al. 2006, Sanabani et al. 2006). B19V genotype 1 DNAnemia has also been described after transplantation of B19V DNA-positive kidneys (Barzon et al. 2009) and after bone marrow transplantations (Söderlund et al. 1997b). Whether the viremic events obtained after transplantation represent primary infections, exogenous re-infections, or reactivation is often difficult to determine due to the immunosuppressive status of transplant recipients.

Pozzuto et al. (2011) described the possible mechanism for B19V reactivation *in vitro*. They infected non-permissive endothelial hybrid cells (Eahy926) and 293 cells with B19V and observed activation of B19V protein expression after infection with adenovirus (Pozzuto et al. 2011). Furthermore, the adenovirus infection induced the appearance of putative replication intermediates of B19V. It might be that another virus with potential transactivation functions can activate persistent B19V. If reactivation of this type occurs *in vivo,* and if it has any pathological role or is immediately neutralized by the host immune system, are the questions to be answered in the future.

Rapid sequence changes of B19V (III)

The wide and different distribution, prevalence, and existence of B19V genotypes have awakened interest in the evolution and molecular epidemiology of this virus. Large dataset of B19V genotype 1 VP1/VP2 sequences of plasma-derived B19V viruses with known sampling dates was generated and analyzed. In addition, the dataset of tissue-derived variants of B19V was constructed and compared with plasma-derived data.

At first, synonymous-site variability (SSV) within B19V genotype 1 and between genotypes was examined and suppressed regions overlapping the B19V small proteins were observed. In addition, suppression was observed in a third region, between nucleotide positions 2650 and 3200. These suppressed overlapping coding sequences were excluded from the evolution analysis. Next, the phylogenetic analysis (Figure 7.) was carried out from amplified plasma-derived sequences and from sequences obtained from GenBank. Altogether 50 plasma-derived sequences were used for phylogenetic analysis, which showed evidence of time-related sequence drift of B19V from the root of the tree with later sampling dates. Furthermore, regression analysis revealed a sequence change rate of $4x10^{4}$ substitutions/site/year for each sequence. Sequence change rate of synonymous sites showed even higher substitution rate. The same dataset was also analyzed by the Bayesian MCMS method by applying strict and relaxed molecular clocks. Substitution rates of $3.64x10^{-4}$ and $3.72x10^{-4}$ substitutions/site/year were observed. By this method the predicted years for the most recent common ancestor were from 1956 to 1959. These predicted years, in the late 1950s, are consistent with the epidemiology of B19V genotypes 1 and 2. As shown in epidemiological Study II, with a large number of human tissues and sera, the genotype 1 infections are detected in contemporary samples with extremely few genotype 2 infections. Genotype 2 was prevalent in tissues of subjects born before 1973. In other words, the most recent common ancestor for B19V genotype 1 in the 1950s, in combination with the Study II with tissue samples, supports the assumption that the currently circulating genotype 1 is actually a new B19V variant. The later-identified B19V genotype 2 is actually an older variant that indeed has disappeared from wide circulation.

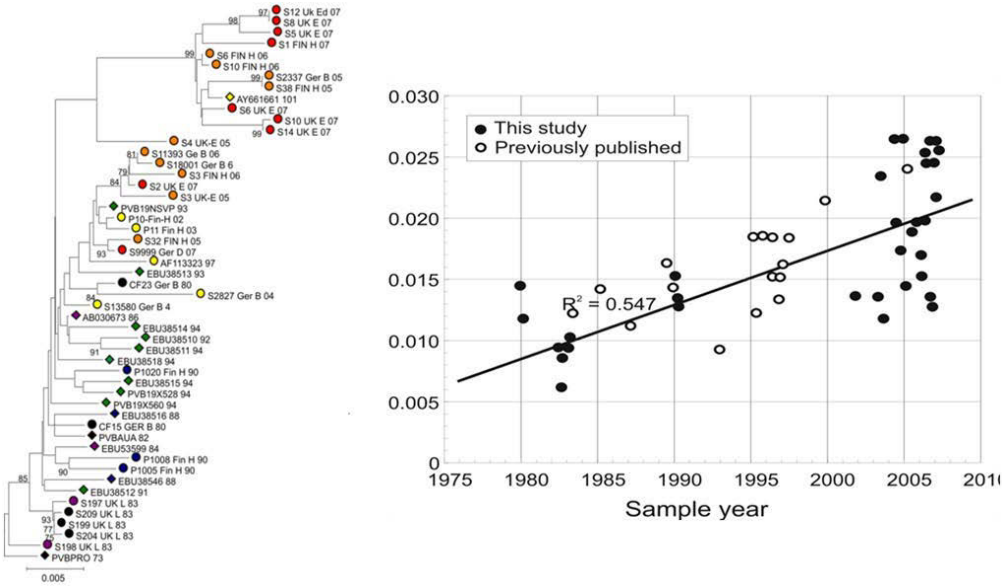

Figure 7*. Phylogenetic analysis (left) and relationship between sample year and divergence between the sequence of the reference B19V strain collected in 1973 (right).*

To investigate whether persistent B19V evolved in a comparable manner to the plasma-derived B19V, a phylogenetic tree was also constructed from tissue-derived B19V sequences. The tree lacked the temporal structure. Furthermore, the correlation between sample dates and divergence was also lacking (Figure 8.). The substitution rates calculated for the tissue-derived variants by MCMC method were 10-fold lower than those calculated for the plasma-derived viruses.

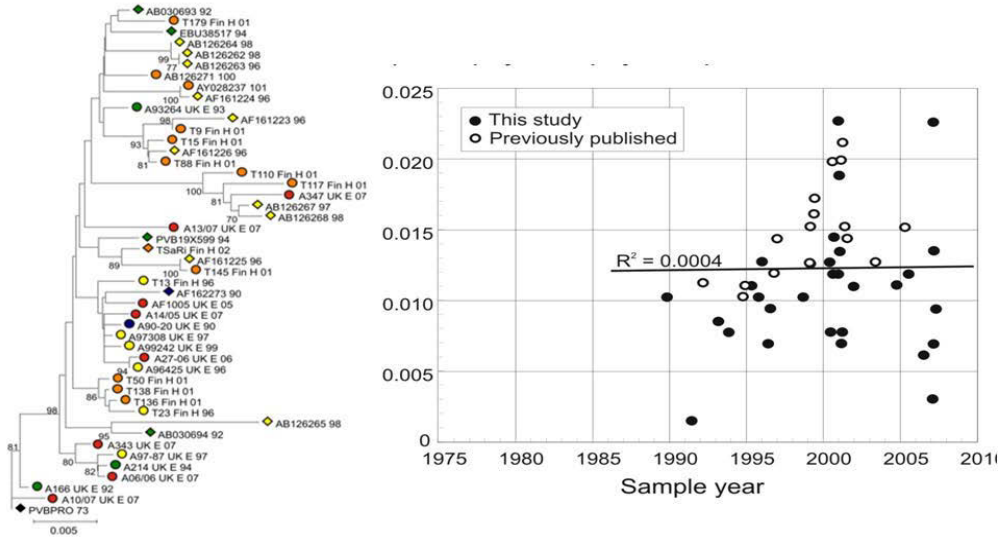

***Figure 8**. Phylogenetic analysis (left) and the relationship between sample year and divergence, between the sequence of the reference B19V strain collected in 1973 (right).*

The observed substitution rates from plasma-derived sequences by both the regression and MCMC analyses were approximately fourfold higher than those observed earlier for B19V genotypes 1 and 3 (Shackelton et al. 2006b, Parsyan et al. 2007). This may possibly be due to analysis of tissue-derived sequences. It was shown that the sequence changes in B19V variants during persistence are not modeled adequately by assuming that they have the same substitution rate as horizontally transmitted viruses. The effect of the inclusion of the tissue-derived sequences was also demonstrated by analyzing combined data sets of plasma- and tissue-derived B19V variants by the MCMC method and obtained a substitution rate of 1.66×10^{-4}. This was three times lower than the rate for plasma-derived viruses. Previous analyses by Shackelton et al. (2006b) and Parsyan et al. (2007) also included regions with suppressed variability, which were shown by us to effectively drive the SSV down close to zero.

It has been shown that sequence changes in B19V genome occur at the synonymous sites (Shackelton et al. 2006b, Parsyan et al. 2007, Norja et al. 2008). When comparing the synonymous substitution rate of B19V with the substitution rates of other viruses, the rate 1.8×10^{-3} synonymous substitutions/site/year of B19V lies within the range of flaviviruses, paramyxoviruses, and orthomyxoviruses, and was even higher than reported for togaviruses and reoviruses. The evolution rates within B19V NS1 and VP areas are similar (Shackelton et al. 2006b, Parsyan et al. 2007), which suggest that B19V

evolution is not driven primarily by immune pressure. Furthermore, the association of a high diversity of genomes with low amino acid variability is in line with the results of similar pathogenicity and antigenic reactivity between genotypes (Nguyen et al. 1999, Blûmel et al. 2005, Candotti et al 2006, Parsyan et al. 2006, Ekman et al. 2007) Whether the rapid evolution rates could have affected distributions of B19V genotypes or disappearance of genotype 2 from circulation, is not known. Within the carnivore parvovirus clade, the cessation of CPV2 and its replacement by the more effective lineage, CPV2a, has been described, and resembles a situation observed for B19V genotypes 1 and 2. Within the CPV strains, the capsid protein gene is under positive selection, most likely due to the driving force of host adaptation and immune escape (Hoelzer et al. 2008). Similar calculations for B19V genotypes 1 and 2 have not been performed, which is due to the lack of B19V genotype 2 blood-derived sequences. Calculations with the tissue-derived persistent sequences would lead to incorrect results, as we have shown that the sequence changes during persistence are not identical with the blood-derived acute phase viruses. The calculations comparing B19V genotypes 1 and 3 suggest that B19V genotype 3 is actually more ancient than B19V genotypes 1 and 2 (Parsyan et al. 2007, Lukashov and Goudsmit 2001).

The reasons for rapid sequence changes of B19V are not known. Similar evolution rates have been shown for carnivore parvoviruses and porcine parvovirus, which may suggest that high mutation rates are characteristics for all autonomous parvoviruses (Shackelton et al. 2005, Hoelzer et al. 2008, Streck et al. 2011). Although autonomous parvoviruses replicate by using the host cell polymerase, it is possible that the required polymerase, or proofreading proteins, do not repair unique single-stranded viral genomes, which are synthesized in unidirectional way, as accurately as they replicate or repair cellular genomes. In mammalian cells, the rate for synonymous substitutions is approximately $2x10^{-9}$ substitutions/site/year (Bulmer et al. 1991), which is around a million times slower than calculated for B19V genomes. Substitution rates for RNA viruses correlate with the genome size; lower rates occurring in viruses with larger genomes (Jenkins et al. 2002, Duffy et al. 2008). This provides evidence that viruses, in common with larger organisms, have the ability to modulate their mutation rates to provide a correct balance between replicative fitness and adaptability. It is also possible that there is evolutionary trade-off between replication speed and replication fidelity, meaning that high mutation rates are simply consequences of rapid replication (Duffy et al. 2008).

The observation that sequence changes of tissue-associated B19V variants showed no correlation with sample collection dates indicates that the evolution of B19V during its

persistence differs from that of horizontally transmitted viruses. Whether these persistent B19Vs are active or replicating at a low level are questions for future studies.

Conclusions

Two new B19V genotypes were found in 1999 and 2002 from blood and human skin, and have been shown, together with the prototype B19V, to form a single serotype of B19V. This thesis describes the evaluation of two commercial qPCR assays for the detection and differentiation of B19V genotypes (Study I). Both methods were functional in detection of B19V genotype 1 but only one of them was good for detection, quantification, and differentiation of the B19V genotypes 2 and 3.

With the evaluated qPCR assay and qualitative PCRs, the prevalence of B19V genotypes in human blood (Studies I and II) and solid tissues (Study II) was analyzed. B19V genotype 1 was prevalent in blood and tissue samples while genotype 2 was detected only in tissues of subjects born before 1973. Genotype 3 was absent from all the tissues studied. The age distribution of the donors harboring B19V genotypes 1 and 2, together with the absence of genotype 2 in blood, suggested the disappearance of B19V genotype 2 from wide circulation after 1970s (Study II). The total absence of genotype 3 in the samples analyzed in this study indicated a sporadic circulation of this genotype in Northern and Central Europe, which is further supported by other studies in which B19V genotype 3 was detected to be endemic in African and South-American regions.

The presence of B19V DNA in human heart tissues of seropositive subjects was shown not to be associated with pathogenesis (Study V). The lifelong persistence of B19V genomic DNA in human solid tissues after primary infection was found to be a characteristic feature of B19V. The remarkable longevity of B19V persistence, a feature that was named *Bioportfolio,* has interesting potential utilities; at the level of individual patients it provides a registry of individual's infectious encounters. At the epidemiological level it provides a database for the analysis of the occurrences of viruses and their variants.

The rate of parvovirus evolution has been observed to be higher than previously suggested (Study III). The reason for that is not known, but similar evolution rates have also been detected for carnivore parvoviruses and for porcine parvovirus. This suggests that the rapid sequence changes may be typical for parvoviruses and thus could have affected different distribution of B19V genotypes.

The ability of human bocaviruses 1-4, and human parvovirus 4, to occur in human solid tissues was also examined by PCR methods (Study IV). HBoV1 DNA was detected at low prevalence in the tonsils of young children. However, the tissues obtained from seropositive adults, did not contain HBoV1 DNA, indicating a likely slow evanescence of

HBoV1 from the tonsillar tissues of small children rather than long-term persistence. Neither PARV4 DNA nor PARV4 antibodies were detected in the samples. However, PARV4 has been shown to be more prevalent among Finnish IDUs, indicating parenteral infection route for PARV4.

The molecular and cellular mechanisms that maintain B19V persistence are not known. Several possibilities have been described for other parvoviruses, including episomal and endogenous persistence, and integration. The exact mechanism for B19V persistence and mechanism for occurrence of HBoV1 in human tonsillar tissues remains to be answered in future studies.

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